ISSN: 1949-4998 Volume 2, Number 7, July 2010

Scientific Research

Health

ISSN: 1949-4998

Editor-in-Chief
Kuo-Chen Chou

www.scirp.org/journal/health

Journal Editorial Board

ISSN 1949-4998 (Print) ISSN 1949-5005 (Online)

http://www.scirp.org/journal/health

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HEALTH

Journal Information

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Vol.2, No.7, 641-651 (2010)
doi:10.4236/health.2010.27098

Health

Statistical models for predicting number of involved nodes in breast cancer patients

Alok Kumar Dwivedi[1]*, Sada Nand Dwivedi[2], Suryanarayana Deo[3], Rakesh Shukla[1], Elizabeth Kopras[4]

[1]Center for Biostatistical Services, Department of Environmental Health, College of Medicine, University of Cincinnati, Cincinnati, USA; *Corresponding Author: alok_bhu1@yahoo.co.in
[2]Department of Biostatistics, All India Institute of Medical Sciences, New Delhi, India
[3]Department of Surgical Oncology, All India Institute of Medical Sciences, New Delhi, India
[4]Department of Environmental Health, College of Medicine, University of Cincinnati, Cincinnati, USA

Received 12 March 2010; revised 8 April 2010; accepted 10 April 2010.

ABSTRACT

Clinicians need to predict the number of involved nodes in breast cancer patients in order to ascertain severity, prognosis, and design subsequent treatment. The distribution of involved nodes often displays over-dispersion—a larger variability than expected. Until now, the negative binomial model has been used to describe this distribution assuming that over-dispersion is only due to unobserved heterogeneity. The distribution of involved nodes contains a large proportion of excess zeros (negative nodes), which can lead to over-dispersion. In this situation, alternative models may better account for over-dispersion due to excess zeros. This study examines data from 1152 patients who underwent axillary dissections in a tertiary hospital in India during January 1993-January 2005. We fit and compare various count models to test model abilities to predict the number of involved nodes. We also argue for using zero inflated models in such populations where all the excess zeros come from those who have at some risk of the outcome of interest. The negative binomial regression model fits the data better than the Poisson, zero hurdle/inflated Poisson regression models. However, zero hurdle/inflated negative binomial regression models predicted the number of involved nodes much more accurately than the negative binomial model. This suggests that the number of involved nodes displays excess variability not only due to unobserved heterogeneity but also due to excess negative nodes in the data set. In this analysis, only skin changes and primary site were associated with negative nodes whereas parity, skin changes, primary site and size of tumor were associated with a greater number of involved nodes. In case of near equal performances, the zero inflated negative binomial model should be preferred over the hurdle model in describing the nodal frequency because it provides an estimate of negative nodes that are at "high-risk" of nodal involvement.

Keywords: Nodal Involvement; Count Models; Breast Cancer

1. INTRODUCTION

Accurate prediction of the number of involved nodes in breast cancer patients helps in grading severity of disease, avoid extensive axillary surgery dissections and assists with treatment decisions such as the use of neoadjuvant chemotherapy [1,2]. Many studies have been performed to predict nodal status in breast cancer patients. Most of them merely predict the presence/absence of involved nodes rather than the number of involved nodes [3]. Until now, only two studies have tried to predict the number of involved nodes in breast cancer patients. Guern and Vinh-Hung [3] found that a negative binomial model describes the number of nodal involvement better than the Poisson model due to excess variability, a condition called over-dispersion. Another study showed that the negative binomial model provides a better fit as compared to the Poisson model for the total number of involved nodes in breast cancer patients in a meta-analysis [4]. These studies used a negative binomial model, which posited that the over-dispersion occurred entirely due to unobserved heterogeneity and/or nodal clustering.

However, count data often involve over-dispersion not only due to unobserved heterogeneity and/or clustering but also due to the preponderance of zero frequency (negative node in the case of cancer) [5]. Consequently, the nominal Poisson or the negative binomial distributions may not satisfactorily account for excess variability if this variability is indeed due to excess zeros. In such situations, use of these models may likely underestimate the probability of negative node status, and may provide misleading results. Zero hurdle or zero inflated regression models can be used to increase predictability in situations with excess zeros.

In count data, the observed zeros can be either structural zeros (e.g., the subject is at no risk of the event of interest) or sampling zeros (e.g., the subject is indeed at some risk of the event of interest). It has been suggested that zero hurdle models are more appropriate in case of excessive sampling zeros while zero inflated models should be preferred in cases of mixtures of zeros *i.e.*, involvement of both types of zeros [6]. In breast cancer, all the patients are indeed at some risk of having nodal involvement and thus all zeros are strictly sampling zeros. Thus, according to the prevailing wisdom, zero hurdle models could be employed to predict the nodal frequency among breast cancer patients.

In epidemiologic studies, generally count data involves zeros at some risk of outcome of interest. In such circumstances, there exists alternative ways to conceptualize the so-called structural zeros and sampling zeros. Using the epidemiological parlance, we can conceptualize zeros in terms of disease *on-set* and disease *progression*. In breast cancer patients, a lack of nodal involvement (observed zero) may be because the cancer is detected early enough in the disease progression (closer to the time of disease onset) or the cancer itself is of slow progression and/or absence of risk factors for high rate of disease progression. These kinds of zeros may be identified as true or structural zeros. The rest of the zeros may be observed in the presence of various risk factors leading up to a high rate of disease progression. These latter types of zeros can be identified as false or sampling zeros. Thus, within the framework of zero inflated models, excess zeros can be modeled as a mixture of true zeros and false zeros. Note that the false zeros can also arise either due to chance, false recording and/or due to false observation. It has been reported that some of the involved (positive) nodes may be recorded as negative due to misclassification by the pathologist (referred to as reporting error) [7]. One study reported that non-dissection of complete axillary lymph nodes might provide false negative nodes [8]. These false negative nodes may be more likely to be found among patients with a high risk of nodal involvement. This indicates a need of estimation of false negative nodes so that they can follow up or be reassessed for diagnostic accuracy. In these situations, we suggest use of the zero inflated models, not only to account for excess zeros, but also to estimate the proportion of false zeros or patients with zeros at high risk of nodal positivity.

Significant applications of zero hurdle and zero inflated models have been made in various fields of research [9-11]. In recent years, the application of these models and their comparisons with other count models has also increased in medical and health fields [12-19]. A review of the application of such models in health research is also reported [20]. Extensions of these models for describing correlated data have also been reported [21-24]. These studies illustrate that zero hurdle/inflated models should be used if over-dispersion in the data is due to excess zeros. Results also indicate that zero hurdle models should be preferred if only at-risk zeros are present in the population. However, to our knowledge, the relative performance of zero hurdle and inflated models in predicting the number of involved nodes has not been addressed. In this paper, prediction of the number of involved nodes is made using Poisson regression (PR), negative binomial (NB), zero hurdle Poisson (ZHP), zero inflated Poisson (ZIP), zero hurdle negative binomial (ZHNB) and zero inflated negative binomial (ZINB) models. Zero hurdle models in many epidemiologic studies like the present one may satisfactorily account for excess zeros, perhaps even as good as zero inflated models. We arguably demonstrate that the zero inflated models have an added advantage over the former in describing the event of interest in relation to the disease process itself, including identification of the factors involved in predicting the disease onset and disease progression.

2. MATERIALS AND METHODS

2.1. Subjects

We utilized one of the largest breast cancer datasets available in India to assess the number of involved nodes distribution. The data were extracted from the computerized database of breast cancer patients maintained at the Department of Surgical Oncology, Institute Rotary Cancer Hospital (IRCH), All India Institute of Medical Sciences (AIIMS), New Delhi, India, a tertiary care center, during the period from January 1993 to January 2005. The dataset was updated using the original records kept in the record section of IRCH. Data from all patients who underwent surgery for breast cancer, including axillary lymph node dissections, were included in this study. Patients with recurrent breast cancer, bilateral breast carcinoma, any evidence of metastasis, unknown primary site and male breast carcinoma were excluded from the study.

Covariates and their forms were chosen based on breast cancer literature and an exploratory analysis of this dataset.Patients' age at presentation was stratified as younger (below 35 years) and elder (more than or equal to 35 years). Duration from onset of symptoms until presentation was classified as less than or equal to 2, 2-4, 4-8 and more than 8 months. Parity was categorized as nulliparous, single/doubleparous, and multiparous. Other covariates included menopausal status (post/pre); family history of breast cancer (absent/present); primary side (left/right); skin changes (no/yes); neoadjuvant chemotherapy (no/yes); primary site {medial (lower inner quadrant and upper inner quadrant)/lateral (lower outer quadrant and upper outer quadrant)/central (multiple, central and others)}; tumor type (infiltrating ductal carcinoma/infiltrating lobular carcinoma and others); and pathological tumor size was according to TNM classification (< = 2/2-5/> 5cm). The neoadjuvant chemotherapy and total number of dissected nodes were only used in the model for adjustment, because these variables are highly associated with involved nodes. The study population consisted of all cases of breast cancer and the outcome in question was the number of involved nodes in a patient. Patients with negative nodes (zeros) were divided into two groups-those with "at low risk" of nodal involvement and those with "at high risk" of nodal involvement. A patient with negative nodes and having a relatively low risk of nodal involvement was defined as "at low risk" zero and labeled, in the context of modeling, as a "true or structural" zero. The remaining patients with negative nodes and a relatively high risk of nodal involvement due to the presence of various risk factors were defined as "at high risk" zeros. In the context of modeling, we label them as "false or sampling" zeros.

2.2. Statistical Models

The Poisson regression model (PR) describes count outcomes or proportion/rates. Generally, the PR model explains less variability of counts than the observed variability. As a result, this often gives misleading relationships between covariates and outcomes. Excess variability can be adjusted within the PR framework using inflation approaches of standard errors of the regression coefficients [25]. As such, it may be the appropriate model to use for drawing correct inferences in the case of over-dispersion due to unobserved heterogeneity and/or clustering/temporal dependency. However, it may not be the most appropriate in the case of excess zeros, as expected in assessing the distribution of number of involved nodes. In the PR model, y_i is the number of involved nodes for the i^{th} patient, and λ_i is the mean number of involved nodes. If the number of involved nodes follows a Poisson distribution, its probability mass function can be expressed as:

$$f\left(y_i|x_i\right)=\frac{e^{-\lambda_i}\lambda_i^{y_i}}{y_i!},\quad y_i=0,1,2\ldots,i=1,2,....n,\lambda_i>0 \quad (1)$$

If β_i's are regression coefficients corresponding to the set of considered covariates x_i's, and k is the number of considered covariates, then the PR model can be expressed using **Eq.1** as:

$$\log\left(\lambda_i\right)=\beta_0+\beta_1x_1+\beta_2x_2+\cdots+\beta_kx_k \quad (2)$$

As an alternative to the PR model, the negative binomial (NB) model has an inbuilt provision to account for over-dispersion due to unobserved heterogeneity and/or temporal dependency [26]. As a result, this model helps not only in adjusting the standard errors of the regression coefficients but also provides a more flexible approach for prediction of the count outcome. Under the assumption of over-dispersion being merely due to unobserved heterogeneity and/or temporal dependency, the NB model was used. The unobserved heterogeneity may be due to unobserved predictors and/or too much variation in some of the clinical and pathological cofactors. Temporal dependency in nodes may be occurring due to clustering of nodal involvement within patients. The NB model is expressed as:

$$f\left(y_i|x_i\right)=\frac{\Gamma(y_i+\alpha^{-1})}{\Gamma(y_i+1)\Gamma(\alpha^{-1})}\left(\frac{\alpha^{-1}}{\alpha^{-1}+\lambda_i}\right)^{1/\alpha}\left(\frac{\lambda_i}{\alpha^{-1}+\lambda_i}\right)^{y_i}, \quad (3)$$
$$y_i=0,1,2.....;i=1,2.....n;$$

In this model, α is the over-dispersion parameter due to unobserved heterogeneity and λ_i is the mean number of involved nodes. The NB regression model can be obtained similar to **Eq.2** by using **Eq.3**.

The NB model may not be appropriate if the over-dispersion is due to excess zeros because it underestimates the probability of zeros and consequently underestimates the variability present in the outcome. In such situations, alternative models such as zero inflated/hurdle models that account for over-dispersion due to excess zeros are useful.

Zero hurdle models are typically used when the excess zeros arise from an "at risk" population. Under the assumption that over-dispersion results from excess zeros arising from an "at risk" group, zero hurdle Poisson (ZHP) was used. In this model, all zeros are considered to be observed from a non-counting process, as opposed to a counting process. Within this model, all zeros are typically described through logistic regression, whereas positive counts are described through a zero truncated

Poisson model. In the ZHP model, p_i is "at risk" negative nodes under logistic model. Assuming the mean number of involved nodes (λ_i) under zero truncated Poisson model, the ZHP distribution may be expressed [27] as:

If γ_i's and β_i's are respective regression coefficients under logistic and zero truncated Poisson models corresponding to considered covariates (x_i's), and the number of considered covariates is k in each of the models, then using **Eq.4** regression models can be expressed as:

$$\log\left(\frac{p_i}{1\text{-}p_i}\right)=\gamma_0+\gamma_1x_1+\gamma_2x_2+\cdots+\gamma_kx_k$$
$$\log(\lambda_i)=\beta_0+\beta_1x_1+\beta_2x_2+\cdots+\beta_kx_k \tag{5}$$

The ZHP model provides two sets of results. These results can also be obtained separately by fitting both a logistic regression and zero truncated Poisson model. This is why hurdle models are referred to as two-part models. The binary process model identifies factors associated with the presence/absence of nodal involvement, whereas modeling count process yields factors associated with an increase in the number of involved nodes given that the patient has involved nodes. Note that the ZHP model accounts for over-dispersion due to excess zeros but not due to unobserved heterogeneity and/or temporal dependency in nodal involvement. In the latter case, one may use the zero hurdle negative binomial (ZHNB) model by considering count process as zero truncated negative binomial distribution. Substituting a zero truncated negative binomial distribution in **Eq.4** yields the ZHNB distribution, and it can be expressed as **Eq.6**.

Zero inflated models are typically used when the excess zeros are a mixture of two types of zeros-true (structural zeros) and false (sampling zeros). We propose to categorize the negative nodes in our population as a mixture of two types, those with very low/no risk of nodal involvement (true zeros) and those with high risk of nodal involvement (false zeros). In this way, use of the zero inflated model framework not only accounts for the extra variability due to excess zeros but also estimates the relative proportion of these at "low risk" and at "high risk" zeros. Further, this can be used to identify subjects with a high likelihood of being in one or the other type of zero classification using the risk factors. In zero inflated models, occurrence of zeros is considered as a result of two distinct processes. Some of the zeros (zeros at "high risk") are considered to be observed from counting process and others (zeros at "low risk") from non-counting process. As an inbuilt mechanism within these models, true zeros are typically described through logistic regression, whereas false zeros are described through simple count model. Like hurdle models, the zero inflated models also provide two sets of results. However, the interpretation of regression coefficients under inflated models is different from the hurdle models. Modeling binary process provides factors associated with negative nodes in a "low risk" population as compared to a "high risk" population, whereas modeling count process provides factors associated with the extent of the number of involved nodes, including false negative nodes given that patients are in a high risk population. Here, the probability of observing negative nodes is the sum of observing negative nodes (true) under the logistic model plus the probability that a individual is not in the binary process, and the probability that negative nodes (false) under the considered count model. If the count process follows the Poisson distribution then it is called a zero inflated Poisson (ZIP) model. To understand the ZIP model, consider the occurrence of at "low risk" negative nodes with probability p_i under a logistic model, whereas that of involved nodes (including at "high risk" false negative nodes) with probability (1-p_i) under the Poisson model, having a mean number of involved nodes (λ_i,), the ZIP distribution can be expressed [28] as:

$$f(y_i|x_i)=\begin{cases}p_i+(1-p_i)\exp(-\lambda_i), y_i=0\\ (1-p_i)\dfrac{\exp(-\lambda_i)\lambda_i^{y_i}}{\Gamma(y_i)}, y_i\ge 1; 0\le p_i\le 1; \lambda_i>0\end{cases} \tag{7}$$

$$f(y_i|x_i)=\begin{cases}p_i & , \ y_i=0\\ (1-p_i)\dfrac{\exp(-\lambda_i)\lambda_i^{y_i}}{y_i!(1-\exp(-\lambda_i))}, & y_i\ge 1;\ 0\le p_i\le 1;\ \lambda_i>0;\ i=1,2,\ldots,n\end{cases} \tag{4}$$

$$f(y_i|x_i)=\begin{cases}p_i & , \ y_i=0\\ (1-p_i)\dfrac{\Gamma(y_i+\alpha^{-1})}{\left(1-\left(\dfrac{\alpha^{-1}}{\alpha^{-1}+\lambda_i}\right)^{1/\alpha}\right)\Gamma(y_i+1)\Gamma(\alpha^{-1})}\left(\dfrac{\alpha^{-1}}{\alpha^{-1}+\lambda_i}\right)^{1/\alpha}\left(\dfrac{\lambda_i}{\alpha^{-1}+\lambda_i}\right)^{y_i}, & y_i\ge 1\end{cases} \tag{6}$$

If $\gamma_i^{'}s$ and $\beta_i^{'}s$ are respective regression coefficients under logistic and Poisson models corresponding to considered covariates (x_i's), and the number of considered covariates is k in each of the models, then using **Eq.7**, regression models can be expressed as:

$$\begin{aligned} \log\left(\frac{p_i}{1-p_i}\right) &= \gamma_0 + \gamma_1 x_1 + \gamma_2 x_2 + \cdots + \gamma_k x_k \\ \log(\lambda_i) &= \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_k x_k \end{aligned} \quad (8)$$

If the count process does not follow the Poisson model then one may use the zero inflated negative binomial (ZINB) model by considering count process as a negative binomial distribution. In contrast to ZIP, the ZINB model accounts for the over-dispersion due to both types of zeros as well as due to unobserved heterogeneity and/or temporal dependency. Substituting negative binomial distribution in **Eq.7**, the ZINB distribution can be expressed as:

2.3. Model Comparisons

The PR, NB, ZIP, ZHP, ZHNB and ZINB models were used to describe the number of involved nodes in breast cancer patients. The covariates found to be significant in univariate analysis with any of the regressions were included into all the regression models to maintain the comparative findings. The nested models (e.g., PR versus NB and ZIP, NB versus ZINB, and ZHP versus ZHNB) were compared using a likelihood ratio. Significant result of the likelihood ratio test of comparison (PR versus NB, NB versus ZINB, and ZHP versus ZHNB) indicates the presence of over-dispersion due to heterogeneity and/or temporal dependency. The non-nested models (PR with ZHP, PR with ZHNB, PR with ZINB, NB with ZHP, NB with ZIP, NB with ZHNB, ZHP with ZIP, ZHP with ZINB and ZHNB with ZINB) as well as nested models were also compared using the Vuong test [29]. Significant and better fit of comparisons (PR with ZHP/ZIP, and NB with ZHNB/ZINB) explores whether or not the over-dispersion is due to excess zeros.

To compare the predictive performance of the models, various indices such as log likelihood, Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), mean squared prediction error (MSPE) and mean absolute prediction error (MAPE) were also obtained. A probability plot (observed probability minus predicted probability of positive nodes versus number of positive nodes) was constructed for each model. The probability plot was constructed after truncation at 10 positive nodes for ease of visual comparison. The best-fitted model was also validated using the leave-one-out cross validation method [30]. The p-values less than 5% were considered as significant results. STATA 9.0 package was used for all statistical analyses.

3. RESULTS

A total of 1152 patients were found to be eligible for this study. Of those in the study, the presence of involved nodes was found in 705 (61.2%) patients. The mean and standard deviation of the number of involved nodes per patient were 3.9 and 5.6 respectively (median 1 and range: 0-33). Median number of total dissected nodes per patient was 14 (range: 1-46). The mean age was 47.7 (standard deviation, 11.1) years and range 20-86 years. The distributions of covariates considered in the analysis are shown in **Table 1**.

A descriptive comparison reveals that the cofactors parity, skin changes, primary site and pathological tumor size were consistently associated with outcome across all models. Three additional covariates, age, menopausal status and tumor type, were statistically significant only in the PR model. There was good concordance in the assessment of statistical significance in all aspects among ZHP, ZIP and NB models. A similar relation could also be seen between the ZINB and ZHNB models in providing factors associated with the extent of nodal involvement. In other words, parity, skin changes, primary site and tumor size were found associated with a greater number of involved nodes in both models. However, the ZHNB model provided primary site, skin changes and pathological tumor size associated with presence of positive nodes whereas ZINB model provided only primary site and skin changes associated with presence of positive nodes in at high-risk population.

The significant Pearson chi square goodness of fit (gof) test ($p < 0.001$) along with other characteristics of model fit indicated that the PR model produced a poor fit for nodal involvement data. In the NB model, the estimated dispersion statistic (α) was 1.73 (95% CI: 1.54, 1.95). A significant likelihood ratio test ($p < 0.001$) of dispersion

$$p(y_i|x_i) = \begin{cases} p_i + (1-p_i)\left(\dfrac{\alpha^{-1}}{\alpha^{-1}+\lambda_i}\right)^{1/\alpha} & , y_i = 0 \\ (1-p_i)\dfrac{\Gamma(y_i+\alpha^{-1})}{\Gamma(y_i+1)\Gamma(\alpha^{-1})}\left(\dfrac{\alpha^{-1}}{\alpha^{-1}+\lambda_i}\right)^{1/\alpha}\left(\dfrac{\lambda_i}{\alpha^{-1}+\lambda_i}\right)^{y_i} & , y_i \geq 1 \end{cases} \quad (9)$$

Table 1. Zero inflated negative binomial model for number of involved nodes.

Variables	N	Logistic Portion* Odds Ratio (95% CI)	NB Portion Risk Ratio (95% CI)
Age (year)			
> 35	977	1.00	1.00
< = 35	175	0.98 (0.54, 1.80)	1.12 (0.90, 1.38)
Symptom duration (month)			
< = 2	376	1.00	1.00
3-4	263	0.74 (0.43, 1.26)	1.00 (0.82, 1.23)
5-8	266	1.13 (0.71, 1.81)	1.17 (0.95, 1.43)
> = 9	247	0.73 (0.43, 1.24)	1.08 (0.88, 1.33)
Parity			
Nulliparous	47	1.00	1.00
P1/P2	445	1.18 (0.26, 5.31)	1.82 (1.20, 2.77)
Multiparous	660	1.67 (0.38, 7.44)	1.95 (1.29, 2.95)
Menopausal			
Post Menopausal	587	1.00	1.00
Pre Menopausal	565	0.69 (0.45, 1.04)	1.01 (0.85, 1.18)
Primary side			
Left	583	1.00	1.00
Right	569	0.87 (0.60, 1.26)	0.91 (0.79, 1.06)
Primary site			
Medial (UIQ + LIQ)	235	1.00	1.00
Lateral (LOQ + UOQ)	681	0.62 (0.40, 0.96)	1.29 (1.05, 1.60)
Central/Multiple/Other	236	0.38 (0.19, 0.74)	1.24 (0.97, 1.58)
Skin changes			
No	746	1.00	1.00
Yes	406	0.38 (0.23, 0.62)	1.40 (1.19, 1.66)
Tumor type			
Other/ILC	78	1.00	1.00
IDC	1074	0.62 (0.31, 1.22)	1.14 (0.82, 1.57)
Tumor size (centimeter)			
< = 2	236	1.00	1.00
2-5	666	0.63 (0.40, 1.01)	1.28 (1.03, 1.59)
> 5	250	0.61 (0.34, 1.09)	1.49 (1.17, 1.91)

*The odds ratio of negative nodes in low risk group
All the results are adjusted in relation to neoadjuvant chemotherapy as well as total number of dissected nodes

statistic from zero favored the NB model over the PR model. Recall that more than one third of the patients had negative nodes, indicating an excess of negative nodes. Intuitively, this suggests that over-dispersion is most likely due to excess negative nodes. Firstly, all negative nodes were considered to arise from an at-risk group, justifying use of the ZHP model. Further, to estimate false negative nodes, it was considered that some of these negative nodes might be observed among patients who had a "low risk" of nodal positivity (true zeros) and some proportion might be observed among patients who had "high risk" of nodal involvement (false zeros). With this more natural consideration, the ZIP model was used. Both the Vuong test ($V = 12.60$ and $p < = 0.001$) and the significant likelihood ratio test favored the ZHP model over the PR model. However, the comparison of ZHP and ZIP using Vuong test ($V = 2.01$ and $p = 0.04$) slightly favored the ZIP model. The results of

Vuong tests also favored the NB model over the ZHP model (8.86, p = < 0.001) and the ZIP model (8.84, p < 0.001). As observed through improved fit of the NB model over PR and ZHP/ZIP models, it clearly indicates that over-dispersion is involved due to unobserved heterogeneity and/or clustering. In addition, ZHP/ZIP provided evidence of over-dispersion due to excess negative nodes, in comparison to the PR model. Hence, a model incorporating over-dispersion due to excess negative nodes as well as unobserved heterogeneity simultaneously was expected to provide improved predictability of number of involved nodes. Accordingly, ZHNB and ZINB models were used to predict number of involved nodes. Under ZHNB and ZINB models, the estimated dispersion parameters of zero truncated negative binomial and NB models were observed different than zero as [(α = 0.70; 95% CI: (0.56, 0.87)] and [(α = 0.71; 95% CI: (0.57, 0.89)] respectively. This suggests that ZHNB/ZINB models are more appropriate than ZHP/ZIP models in describing the number of involved nodes. The better fit of ZHNB/ZINB models over the NB model suggests that over-dispersion is not only due to excessive negative nodes but also due to unobserved heterogeneity and/or clustering. The result of the Vuong test showed no difference between ZHNB and ZINB models in predicting nodal frequency (1.53, p = 0.13).

The model fit characteristics are shown in **Table 2**. The minimum BIC was observed for the NB model, followed by ZHNB/ZINB models. However, other validity indices of the model (maximum log likelihood, minimum AIC, MSPE and MAPE) favored ZHNB/ZINB models over all other models. The plot of observed minus predicted probability of involved nodes at each count is shown in **Figure 1**. The PR model underestimates probability of occurrence of negative node and overestimates occurrence of one positive node. The line of difference between observed minus predicted probability of positive nodes was close to the reference zero line, showing better fit of ZHNB/ZINB models than the other models. There is virtually no difference between ZHNB and ZINB models in all aspects of describing the number of involved nodes. The ZINB model provides slightly smaller validity indices as compared to ZHNB. Finally, the ZINB model was assessed by the leave one out cross validation method. The MSPE in cross validation of the ZINB model was the lowest of all the models (0.0007), indicating that the ZINB model performs well for predicting nodal involvement in future patients. The ZINB model predicts that 70.6% all negative nodes are at "low risk" zeros, and the remaining 29.4% are at "high risk" for negative nodes. This indicates that almost 30% of the patients observed as negative for nodal involvement are at "high risk" of nodal involvement based on cofactors.

Table 1 displays the estimates of regression coefficients for various cofactors of both portions of the ZINB model. For ZINB, the results of both parts of the models together help in understanding the role of the factors on nodal distribution. The logistic portion showed that medial primary site and absence of skin changes significantly increased the chance of negative nodes in breast cancer patients. Negative binomial portion reveals that the risk of a greater number of involved nodes was 82 percent higher in single/doubleparous patients versus nulliparous patients, given that the patients are in a high-risk group. Further, this was 95 percent higher among multiparous patients. The patients with lateral site involvement had 1.29 times higher likelihood for having a larger number of positive nodes than patients with the medial site. Women with skin changes had 1.39 times more involvement of higher positive nodes as compared

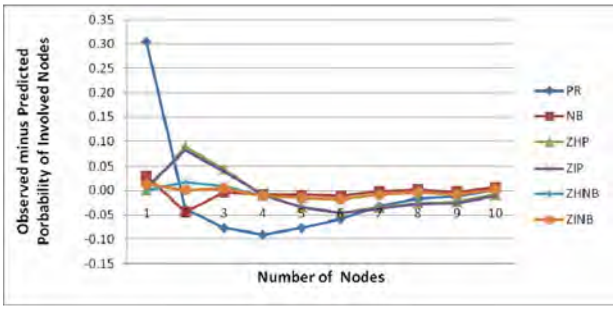

Figure 1. Plots of observed minus predicted probability of positive nodes versus number of positive nodes for six models.

Table 2. Comparison of model fit characteristics.

	PR	NB	ZHP	ZIP	ZHNB	ZINB
Log Likelihood	–4093.9	–2598.6	–3019.7	–3018.4	–2553.7	–2551.1
AIC	8221.8	5233.1	6107.4	6104.8	5185.4	5172.2
BIC	8307.6	5324.0	6279.0	6276.5	5382.3	5348.9
MSPE	4764.0	139.1	632.5	627.62	52.9	49.2
MAPE	27.5	6.2	13.1	13.0	4.8	4.7

to their counterparts. The chance of increased positive nodes was 28 percent higher among patients with 2-5 cm tumor size, in comparison to patients with less than 2 cm tumor size. It was again 1.49 times more likely among patients with more than 5 cm tumor size as compared to less than 2 cm tumor size.

4. DISCUSSION

The number of involved nodes is one of the most important therapeutic and prognostic factors for breast cancer [1]. Clinicians need to predict the number of involved nodes in breast cancer patients in order to improve health outcomes. To the best of our knowledge, few studies have described the number of involved nodes in breast cancer patients, and tested statistical models to accurately predict involved node number. As for most of the count data, studies also found excess variability in nodal distribution than that expected by a Poisson model. They also generally assume the cause of over-dispersion to be solely due to unobserved heterogeneity, and therefore used the NB model to fit and describe nodal frequency [3,4]. However, data with nodal involvement often involve excess zeros, which also cause over-dispersion. This indicates a need to explore fitting zero hurdle and zero inflated models, which can also account for variability due to excessive zeros. In the current paper, we fitted various count models to identify putative causes of over-dispersion, and to assess the predictive performance of these models with regard to the nodal status in a population of patients with breast cancer. We also illustrated the significance of using zero inflated models in count data involving zeros that emanate from the subjects that are all "at-risk" of the event of interest.

The ZHNB/ZINB regression models provide the best fit when predicting the number of involved nodes in breast cancer patients. This confirms that the distribution of the involved nodes contained over-dispersion not only due to unobserved heterogeneity but also due to excessive negative nodes (zeros). As expected, the PR model had the worst prediction ability for nodal frequency. Accounting only one source of over-dispersion, either due to excessive zeros or due to unobserved heterogeneity, the prediction ability of nodal frequency improved as indicated by NB, ZHP, ZIP models. However, use of ZHNB/ZINB models, which assumes involvement of more than just one source of over-dispersion, provided smaller prediction error.

The ZHNB and ZINB models were consistent and similar for factor-identification in the extent of nodal involvement as well as for prediction of number of positive (involved) nodes. In the current study, we focused on predicting nodal frequency. On that basis, either model can be used to predict number of involved nodes. Due to ease of interpreting the results of ZHNB model, it can be preferred over ZINB model. These findings are supported by Rose *et al*. [6], who also found good concordance between the ZHNB and ZINB models on vaccine adverse data—a case of only "at risk" zeros similar to the data used in our study. They suggested that the model selection should be determined based on study objectives and the data generating process. They recommend using the ZHNB model due to involvement of only "at risk" zeros. However, Baughman [31] suggested that model choice should be based on the rationale behind the consideration of data generating mechanism. Gilthorpe *et al*. [32] suggested that the zero inflated models should be used according to the underlying disease process *i.e.*, considerations of disease onset and disease progression. In our opinion, zero hurdle models should be preferred if data consist of zeros which are all coming from the subjects at "no-risk" of the outcome of interest, and over-dispersion is due to excess zeros. In such cases, zeros from the "no-risk" population arise from a non-counting process. However, zeros coming from an "at risk" population belong to the count process, thus influencing model choice based on the rationale behind the data generation of the "at risk" population. In the present study, if diagnosis is close to or at disease onset, the risk of finding the event of interest (nodal involvement) would be minimal, whereas if the diagnosis is late and during disease progression, the risk of the event of interest would be relatively high. Previous studies note that the distribution of involved nodes often consists of some proportion of false negative nodes, which may often arise in the "high-risk" group [7,8]. There is ample evidence to consider "at risk" zeros, at least in breast cancer, as a mixture of "low-risk" and "high-risk" zeros, thus, suggesting the use of zero inflated models. Use of the ZINB model not only gives estimate of the false negative nodes *i.e.*, zero at "high risk" of nodal involvement, but also provides slightly better predictive performance than the ZHNB model.

The ZINB model estimated about 30 percent of the zeros that can be considered false/at "high risk" negative nodes, suggesting that these patients are at high risk of nodal involvement. Among these, some patients might have been observed or reported falsely as having negative nodes. If so, then those patients might have been under-treated and/or misclassified, resulting in an inaccurate predicted prognosis. This model will help to identify such patients, and reduce misclassification. There is a need to develop a sound strategy to classify patients at "high risk" zeros and "low risk" zeros. This issue is under investigation by us, and is the subject of a future publication.

The mean square prediction error was found to be 35.4% less using ZINB as compared to the NB regression model. In addition, the predictive performance of the ZINB model was significantly better than the NB regression model, indicating that the NB model may not always be appropriate for describing nodal distribution. The leave-one-out cross-validation assessment of the developed ZINB model provided the minimum mean square prediction error compared to the other developed models, indicating that the model performs well, even for future patients, in comparison to other models.

This study is the first report to analyze patterns of nodal involvement in breast cancer, using a large dataset collected in India. In our study, 61.2% of the patients had the presence of involved nodes. Sandhu *et al.*, using a different Indian dataset, also reported a 61.6% nodal involvement [33]. A different study, also using a population from India, reported an even higher nodal positivity rate of 80.2% [34]. In our study, both presence of other than medial primary site and skin changes among patients are associated with high risk of nodal involvement and with a greater number of involved nodes. In addition to these two factors, higher parity and larger tumor size are also associated with an increased risk of a higher number of involved nodes, given that the patients are in high risk population. These factors are consistently found to be associated with the presence of involved nodes in other studies [35-41], and are directly or indirectly consequences of late diagnosis. Overall, these findings confirm the need for ongoing efforts to minimize diagnostic delay in patients suspected of having breast cancer.

One limitation to our study is that it uses a dataset not designed for our analysis. Important covariates, such as lymphatic vascular invasion and S-phase function, were not included in this database. These covariates could be significantly associated with involved nodes, as reported in various studies [42-45]. In addition, instead of adjustment of these results in relation to dissected number of nodes, an attempt could be made to model the proportion of positive nodes in patients through count data models or binomial models.

5. CONCLUSIONS

The ZHNB/ZINB regression models can be used to describe nodal distribution more appropriately than the NB model. However, the ability of the ZINB model to more accurately estimate at "high-risk" zeros while having a comparatively lower prediction error, as compared to the ZHNB model, suggests that it is the best model for predicting and describing the number of involved nodes. Many of the factors associated with nodal involvement may be a result of diagnostic delay of breast cancer patients, indicating the need to minimize delay in diagnosis of breast cancer patients. There is also a need to further investigate the consequences of using zero inflated models, as an alternative to zero hurdle models, in at- risk populations.

6. ACKNOWLEDGEMENTS

The authors would like to express their thanks to Dr. V. Sreenivas, Department of Biostatistics, All India Institute of Medical Sciences, New Delhi; Dr. Arvind Pandey, National Institute of Medical Statistics, New Delhi; and also Dr. Kishore Chaudhry and Dr. D. K. Shukla, Division of Non-Communicable Diseases, Indian Council of Medical Research, New Delhi, for their critical comments throughout this study.

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Vol.2, No.7, 652-660 (2010)
doi:10.4236/health.2010.27099

Health

PET in uterine malignancies

Valeria Pirro[1], Andrea Skanjeti[1], Ettore Pelosi[2]*

[1]Nuclear Medicine Unit, San Giovanni Battista Hospital, University of Turin, Turin, Italy
[2]PET Centre, IRMET S.p.A., Turin, Italy; *Corresponding Author: e.pelosi@irmet.com

Received 7 March 2010; revised 2 April 2010; accepted 5 May 2010.

ABSTRACT

Positron Emission Tomography (PET) or integrated PET/Computed Tomography (PET/CT) with ^{18}F-Fluoro-Deoxy-Glucose (^{18}F-FDG) is a functional imaging modality, useful in the characterization of undetermined morphological findings, and in the staging/re-staging of a large number of malignancies. Although its use in uterine malignancies has been poorly investigated, in recent years the employment of this technique has constantly increased. In this review, we evaluate the role of PET (/CT) with ^{18}F-FDG in uterine malignancies (cervical and endometrial cancers as well as uterine sarcomas), underlying its advantages and discussing its limitations. Metabolic and anatomic information given by PET/CT with ^{18}F-FDG could be useful in the evaluation of local and distant disease involvement at the staging, in the detection of disease recurrence, and in the evaluation of the response after chemotherapy and/or radio-therapy.

Keywords: ^{18}F-FDG PET/CT; Uterine Malignancies; Cervical Cancer; Endometrial Cancer; Uterine Sarcomas

1. INTRODUCTION

Positron Emission Tomography (PET) or integrated PET/Computed Tomography (PET/CT) with ^{18}F-Fluoro-Deoxy-Glucose (^{18}F-FDG) study is a functional, non invasive whole body examination, that allows to metabolically characterize undetermined morphological findings, stage/re-stage disease, evaluate treatment response and monitor the therapy in a large number of malignancies (lymphomas, lung, breast, colon-rectal cancer, etc). In the genital tract of women the use of PET is controversial and it is limited by the urinary excretion of the ^{18}F-FDG, that interfere with the evaluation of uterus and vagina, and by the numerous false positive findings related to the presence of physiological tracer uptakes in the bowel, ovaries, and in the uterus itself. However, the clinical introduction of integrated PET/CT tomography, allowing the co-registration and the superimposition of anatomical and functional images and thus the exact localization of all the ^{18}F-FDG uptakes, improved PET diagnostic accuracy [1].

The aim of the present review is to discuss the role of ^{18}F-FDG-PET or PET/CT (PET/(CT)) exam in the uterine cervical cancer, in the endometrial adenocarcinoma and in the uterine sarcoma.

2. LITERATURE SEARCH

For this review, in the matter of the role of ^{18}F-FDG-PET (/CT) in the above mentioned gynaecological malignancies, a MEDLINE search has been performed in order to find relevant articles. For all the evaluated malignancies we included only primary studies and meta-analysis published in the English language in the last five years. We did not include case reports, and abstracts. For uterine cervical cancer we used as keywords: uterine cancer, cervical cancer, and uterine cervix carcinoma; while for endometrial cancer we used: uterine corpus carcinoma or neoplasm, and endometrial cancer. Finally, in the case of uterine sarcomas, we used as keywords uterine sarcoma, uterine carcinosarcoma, and uterine leyomiosarcoma. In all of the above mentioned cases, each keyword was always associated with Positron Emission Tomography, PET/CT, and ^{18}F-FDG-PET/CT. Furthermore, to complete the search we look for in the bibliography of the founded studies and considered the most recent and interesting works.

3. CERVICAL CANCER

Cervical cancer is the third most common neoplasm in women. Recent data report an incidence rate of about 42,000 new cases/year in the United States and in the European Union [2,3] and 150,000 deaths/year worldwide [4]. In the last decades, the introduction of the Papanicolau screening test (Pap-test) has allowed an increase in the detection rate of pre-invasive lesions; in the same time the mortality due to the invasive cervical cancer has not substantially decreased [5,6].

To stage cervical cancer the International Federation of Gynaecologists and Obstetrics (FIGO) staging system is currently used [7,8]. In patients with small localized carcinomas (stage IA and IB1) radical hysterectomy or radiotherapy alone are equally recommended [9]. For large lesions and/or locally advanced cancers (stage IB2–IVA), chemo-radiotherapy is the treatment of choice [10,11]. Tumour size, parametrial tissue involvement, pelvic and/or para-aortic lymph node spread and deep invasion of nearby organs are the most important prognostic parameters at the diagnosis [12]. Patients with these unfavourable prognostic factors are at high risk of developing disease recurrence with an estimated recurrence rate ranging between 23% and 35% [13,14].

Even if the assessment of local and distant disease extension is a crucial point both in the pre- and post-treatment phases, however a standardized protocol to stage/re-stage these patients has not been established [15]. In particular, no imaging modalities are routinely used in this work up, which is based on physical examination, Pap-test, serum markers assay and surgical evaluation. Currently, the use of ^{18}F-FDG-PET/CT in the management of patients with cervical cancer has been investigated in different settings.

3.1. Staging

The assessment of the primary lesion is actually based on the clinical examination and on morphological imaging modalities, in particular MRI. One of the crucial data is the presence of uterine parametrial invasion. In this field, despite the presence of co-registered CT images, the diagnostic performance of ^{18}F-FDG-PET/CT is worse than MRI, due to the lack of a good spatial resolution. In the local staging of 32 primary tumours, Park *et al.* highlighted a higher number of false negative results at PET scan than at MRI (3 and 1 case, respectively) [9]. Probably, in stage IA or IB the amount of disease is under or at the limit of the PET system resolution and its detection can be elusive. Moreover, the interference of urinary activity, that of physiological processes (such as hormone-dependent changes in the ovaries and endometrium during the phases of menstrual cycle) and some benign pathologies (such as corpus luteum cysts, endometriosis, inflammations, menstruations, etc.) can interfere with the optimal evaluation of the primary lesion, leading to difficulties in exam interpretation [12,16]. Some of these PET limitations can be reduced with practical expedients: for example, the urinary interference can be avoided by emptying the bladder just before the start of the exam, or by the hydration and administration of diuretics, or by the continuous bladder irrigation to dilute and remove the radioactive urine. On the basis of these limitations, it is clear that, despite the on-going technical improvements, PET is still unsatisfactory in the evaluation of the primary lesion and particularly in the identification of the deep uterine tissue involvement.

On the other hand, an application of PET exam in these cases is actually under debate, *i.e.* the prognostic value of the primary lesion ^{18}F-FDG uptake. The tumour ^{18}F-FDG uptake, generally measured by the maximum Standardized Uptake Value (SUVmax), seems to be strictly related to the behaviour and to the aggressiveness of the tumour itself as in other malignancies (head and neck, lung and oesophageal cancers). In uterine cancer different authors reported an independent correlation between the cancer SUVmax and: the lymph node status, the disease response to chemo/radio therapy, the frequency of pelvic recurrence, the disease free and the overall survival [12,17-20]. In a study on 240 patients, Kidd *et al*. [21] confirmed the correlation between the SUVmax at the staging and the presence of lymph node metastases. Moreover, Lee and colleagues [22] observed a good correlation between SUVmax and DFS in early stages of cervical cancer. From these experiences, it seems that SUVmax could be a useful prognostic tool in this cancer too.

In the pre-treatment disease staging, the identification of nodal (loco-regional and para-aortic) and distant metastasis are crucial points, which present prognostic and therapeutic significance. In fact, in locally advanced cervical cancer the 5 year survival rate is 57%, 34% or 12%, in node negative cases, pelvic nodes metastasis or para-aortic nodes metastasis, respectively [23]. In a recent meta-analysis, the most accurate method to study lymph node involvement resulted to be the sentinel node biopsy; however, this is a (minimal) invasive procedure, that often requires the administration of anaesthetic drugs, and that could lead to some complications. Among the imaging tests, the authors of this meta-analysis affirm that PET/CT presents better accuracy than contrast enhancement (CE) CT and MRI and that it could be used to guide laparoscopic staging procedures [24]. In fact, the sensitivity of CECT and/or MRI in identifying nodal metastasis is very low. A Gynaecological Oncology Group (GOG) study reported a sensitivity of 34% in the detection of para-aortic lymph nodes by CECT [25]. Furthermore, in patients with gynaecological cancer and CECT negative for lymph node metastasis, PET/CT showed sensitivity and specificity of 50% and 83.3% respectively [25]. The good accuracy of PET and PET/CT scans in detecting lymph nodes has been established by the meta-analysis of Havrilesky *et al.*, that assessed a pooled sensitivity and specificity rate of 84% and 95% for para-aortic lymph nodes and 79% and 99% for pelvic lymph nodes [26]. More recently, other authors confirmed these results for PET [27-32]. Furthermore, it was evidenced an increase in the accuracy for combined PET/CT [7,25,33-36]. Finally, Yen *et al*, indicate that an SUVmax of para-aortic lymph nodes greater than 3.3 is a strong negative prognostic factor in patients with locally advanced disease in respect to recurrence and survival rate [36]. On the other hand as reported by several authors, the limit of PET in this field, is represented by the significant number of false negative lymph nodes. This pitfall is related to the limited spatial resolution of the tomograph [31,37-39]. Kitajima *et al.* [40] attempted to improve the accuracy of the exam performing a PET/CECT scan. They found a per patient based sensitivity, specificity, positive predictive value, negative predictive value and accuracy of 50%, 90.9%, 66.7%, 83.3% and 80%, respectively, and per lymph nodes based sensitivity specificity, positive predictive value, negative predictive value and accuracy of 51.1%, 99.8%, 85.2%, 98.9% and 98.7%, respectively. On the other hand, Kim *et al.* [23] advise the use of fused MRI/PET to increase the detection of lymph node metastasis. In conclusion, due to the low sensitivity, the use of PET/CT study to stage lymph nodes should be taken into account only for patients presenting important co-morbidities and/or contraindications to the surgical approach.

3.2. Radiotreatment Planning

Radiation treatment is indicated in a large part of these patients. The accurate definition of the treatment planning is mandatory in order to adequately radiate the tumour and to spare near critical organs. As in other malignancies, the target volume of radiation beam is currently based on morphological examinations (such as CECT and MRI), that allow high spatial resolution images with accurate anatomical definition [41]. However, this staging modality presents some limitations [42] and the adjunct of the metabolic study significantly improve the identification of the target volumes, allowing the identification of the viable part of the tumour, and improving the staging [43,44]. When PET is used, the Planning Target Volume in modified in around 20% of cases [45]. Recently Chao [46] showed the utility of PET/CT in assisting RT treatment planning of patients with potentially curable lymph node metastases. This new information together with the new radiotherapy tools (such as intensity-modulated radiation therapy) allow a decrease in the dose to surrounding healthy tissues, as well as an increase to the target [41,47-50].

3.3. Re-Staging

An early detection and an accurate staging of disease are crucial elements in order to plan the therapeutic strategy and to improve prognosis [51]. In asymptomatic patients previously treated for cervical cancer, the increase of serum levels of markers such as CEA, Ca19.9 and Ca125 is one of the signs of disease recurrence. However, tumour markers are non disease specific and do not give indications about the site of relapse and the amount of disease. In disease re-staging, conventional CECT and MRI are the most frequently used imaging modalities, even if some limitations should be taken into account: first of all, they are generally limited to one body district (pelvis and/or abdomen); secondly, they are often unable to identify cancer relapse in body districts which present post-surgical or post-radiotherapy scars (good sensitivity, but low specificity levels); thirdly, they are inaccurate in characterizing small lymph nodes and in detecting the peritoneal disease [52]. Therefore, as described by van der Weldt [53] the results of the above-mentioned exams are often inconclusive and equivocal, thus justifying the use of PET. In this study the ^{18}F-FDG-PET/CT scan showed a good sensitivity and specificity: 92% and 93% respectively. Furthermore, as reported in **Table 1**, different authors in the recent years investigated the usefulness of PET(/CT) in the suspect of recurrence, and all of them showed high sensitivity and specificity levels. In fact, PET gives a metabolic characterization of the body structures independent to their anatomy, also allowing the investigation of critical regions in which anatomy has been modified [54]. Given this and due to the possibility of identifying distant metastasis, the therapeutic strategies are changed in about 25% of patients after PET exam [26,28].

Table 1. Sensitivity and specificity levels (in percentages) of ^{18}F-FDG-PET(/CT) in the assessment of disease recurrence in cervical cancer.

Authors, year Ref	No. of patients	Sensitivity (%)	Specificity (%)
Sakurai, 2005 [26]	25	91.5	57.1
Lin, 2006 [24]	260	77.3	96.3
Amit, 2006 [24]	28	60	94
Sironi, 2007 [22]	25	93	100
Chung, 2007 [12]	52	90.3	81
Husain, 2007 [25]	20	100	73
Kitajima, 2008 [17]	30	93	93
van der Weldt [53]	40	92	93

As regards the follow-up, Brooks [4] demonstrated that PET/CT presents a good accuracy in detecting recurrences in both asymptomatic and symptomatic women. In his study, it was interestingly showed that the survival of women with asymptomatic recurrences was superior to the symptomatic subjects.

As regards the prognosis, the modifications of the ^{18}F-FDG-uptake during the course of the treatment represents an important predictor of tumour response and patient's survival [55]. Nishiyama [19] evaluated the role of PET in monitoring the neo-adjuvant therapy in patients with advanced stages of gynaecological cancers. In his study, PET showed an accuracy of 85% in predicting response to the treatment, when the SUVmax was decreased more than 65%. However, larger studies are needed to better define the PET role in this context [56].

4. ENDOMETRIAL CANCER

Endometrial cancer is a common malignant disease, being the fourth cancer in post-menopausal women. In Europe, about 1 out of 20 new cancers' cases interests the endometrium and in the United States the incidence rate is about 40,000 new cases/year [57,58]. As in cervical cancer, the surgical-pathological FIGO staging system is used to address patients to the more appropriate treatment. Patients with FIGO stage IA and IB (involvement of less than 50% of myometrium thickness) can be treated with a tumour resection, while patients with stage IC (involvement of more than 50% of myometrium thickness) the para-aortic lymphnode dissection and the use of adjuvant chemo-radiotherapy is one of the proposed therapeutic strategy, due to the fact that this stage has a greater incidence of nodal and distant metastasis, with a worse prognosis [59]. Generally, patients with the clinical suspect of deep myometrial invasion undergo further examinations (CT or MRI), in order to assess the extra uterine disease spread.

The role of ^{18}F-FDG-PET/CT in the management of this neoplasm is not well defined due to the lack of consistent data in the literature, and due to this reason uterine corpus cancer is not included among appropriate applications in oncology [45].

Few small studies assessed the validity of PET alone in the evaluation of primary endometrial cancer, showing good levels of sensitivity (range 83.3-96.7% [59-61]). Torizuka *et al.* affirmed the feasibility of PET study in the assessment of myometrial involvement, reporting a better diagnostic accuracy than MRI (86.4% versus 77.3% respectively) [59]. In fact, in this study the SUV of the tumour correlated significantly with the depth of the tumour invasion. Furthermore, the authors used SUV of the primary tumour, dichotomized to 12, in order to predict the depth of the tumour invasion. They showed that SUV < 12 correlated significantly with superficial invasion, while SUV > 12 correlated with profound invasion of the tumour. However, due to the limited spatial resolution of the PET study, a limited size of invasion could be missed, leading to false negative PET results [62]. Recently, two randomized trials showed that in early stages of endometrial cancer the routine pelvic lymphadenectomy improves the staging, furthermore this surgical staging correlated with prognosis of the patients. However, the advantage gained was only further knowledge, since no survival benefits were observed in these patients. On the contrary, women that undergo surgical staging have increase the risks of complications. Therefore, the goal of the non-invasive staging in these women would be to select those patients in whom the surgical staging could improve prognosis not only the staging [63-65]. In regard to PET/CT, Park *et al.* showed in a population of 53 patients weak levels of sensitivity and specificity in the staging of primary lesions (89.4 and 50.5%, respectively) and in the staging of regional lymph nodes (69.2 and 90.3%, respectively). On the other hand, high accuracy levels were reported in the detection of distant metastasis. The authors concluded that there are two main advantages of ^{18}F-FDG-PET/CT in the preoperative assessment of endometrial cancer: the good negative predictive value in predicting lymph node metastasis, that allows avoidance of surgical staging in poor candidates for such procedure; the high accuracy in detecting distant metastasis [66]. However, again it must be reminded that in the above mentioned study, the negative predictive value (NPV) was good but not excellent (98.9% in the pelvic evaluation, decrease in 87.5% for the para-aortic nodes). Furthermore the NPV was further investigated by Signorelli [63]. It was indicated that in high risk early stages the high NPV could be useful to avoid systemic lymphaedenectomy. In such cases a de-bulking surgery of the involved nodes could be sufficient. With the decrease of lymph node dimension, a progressive significant reduction of sensitivity has been observed by Kitajima *et al.*: 93.3% for lesions greater than 10 mm, 66.7% for lesions between 5 and 9 mm, and 16.7% for lesions smaller than 4 mm [67] Furthermore, Inubashiri [68] observed in recent work that ^{18}F-FDG-PET/CT cannot change the medical management of the patients if a MRI is previously performed.

As indicated by some authors, in endometrial cancer the major contribution of ^{18}F-FDG-PET/CT could be in the early assessment of disease recurrence after therapy [60,69]. The good performance of ^{18}F-FDG-PET/CT has been confirmed by Kitajima *et al.* in a recent paper including 30 patients, that showed an overall patient-based sensitivity, specificity and accuracy of 93% [70].

In conclusion, in endometrial cancer, the most relevant indications obtained by PET are: the possibility of detecting disease recurrence in asymptomatic patients presenting an increase of the Ca125 serum levels; the ability in distinguishing fibrotic tissue from viable lesions after the treatment; the metabolic definition of the radiotherapy treatment planning. Encouraging results are also available in the assessment of lymph nodes and distant metastasis during the disease staging.

5. UTERINE SARCOMAS

Uterine sarcomas are rare neoplastic diseases which represent about a 2-4% of all uterine malignancies [71]. The histology is heterogeneous, but the more represented variety are the leiomyosarcoma, the carcinosarcoma and the endometrial sarcoma. These malignancies present an extremely poor prognosis and, despite their rarity, they are responsible for a large number of deaths every year, in women with uterine cancer. As with the benign uterine leiomyoma, clinical features are represented by vaginal bleeding and pain. Therefore, during the diagnostic process, the differential diagnosis is crucial. Unfortunately, in the majority of cases this is obtained only after surgery at the histopathological examination, because morphological imaging (US and MRI) is often inconclusive.

Therapeutic options in these cancers actually include the surgery (hysterectomy) when the cancer is localized, and chemotherapy if the tumour is locally and/or distantly extended. During the follow-up about one half of Stage I cases develop a recurrence [72]. In the management of sarcomas (soft tissues and bone sarcoma), the current literature evidences discordant data on the accuracy and the usefulness of ^{18}F-FDG-PET scan due to the small number and to the heterogeneity of the included cases; however, PET seems to be capable of differentiating between low and high grade sarcomas, and in the evaluation of residual disease after therapy [73]. In the case of uterine sarcomas, some authors suggest the use of PET study to distinguish between leiomyomas and leiomyosarcomas [74]. The identification of distant metastases could be another reason for the execution of a PET scan in the staging of these patients. However, a recent study presented that only 9% of patients performed an ^{18}F-FDG-PET scan as part of perioperative imaging. [75].

Despite the spare chemotherapy response the precocious and accurate detection of disease recurrence is crucial. However, an effective diagnostic strategy for an early identification of relapse is missing, and specific serological tumour markers are not available. Even if, at this moment, there is not enough evidence to validate the use of this exam in the follow up of asymptomatic patients and in the evaluation of clinically suspected recurrences, in this scenario ^{18}F-FDG-PET/CT could be useful. In fact in a recent study Ho and colleagues [76] showed that PET scan could change the management in falsely inoperable patients or showing distant metastases. On the other hand Park *et al*. [77] showed that the PET scan has a good impact in the surveillance of patients with uterine sarcomas. In fact, in this study, PET scan, show a very good accuracy (94,4%) in women with suspected recurrence and a good sensitivity (87,5%) in asymptomatic women and contribute to change the treatment in 1/3 of the population. The results of the few small reports are encouraging in this way, but not completely in agreement [72,76,77].

Further, clinical studies are needed to define the role of ^{18}F-FDG-PET/CT in uterine sarcomas.

6. CONCLUSIONS

In this review, we evaluated the role of ^{18}F-FDG-PET/CT in uterine malignancies. This safe, non invasive, imaging modality gives useful metabolic and anatomic information, and, as in the other field of oncology, it is beginning to play an important role in the management of these patients. Despite the relatively low number of studies, its usefulness in the assessment of lymph-nodal involvement at the staging, in the detection and staging of disease recurrence, and in the evaluation of the response after chemo- and radio-therapy has been proved. In fact the use of PET/CT in uterine cancer seems to be controversial. Despite numerous and rigorous study demonstrated the utility, gynecologic oncologist are not very enthusiastic of this exam. In fact, such as Kizer *et al*. [78] demonstrated in a recent study, when 83% of them routinely order CT scan, only 28% of them routinely order PET/CT scan. Some of them believe that PET/CT does not provide useful prognostic information whereas others causes could be the difficulty to obtain third party payment from the private paying clients. It seems that the better staging of metastatic lymph nodes or distant metastases, the very good negative predictive value in early stages, the correlation of before/after treatment PET result with the overall survival, data already available, have little impact and do not convince the gynecologic oncologist. However, these data are good sources for the planning of other prospective studies that could have a greater impact.

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ABBREVIATIONS

PET: Positron Emission Tomography;
CT: Computed Tomography;
PET/CT: integrated PET/CT;
CECT: Contrast Enhancement Computed Tomography;
18F-FDG: 18F-Fluoro-Deoxy-Glucose;
Pap-test: Papanicolau screening test;
FIGO: Federation of Gynaecologists and Obstetrics;
MRI: Magnetic Resonance Imaging;
SUVmax: Standardized Uptake Value;
DFS: Disease Free Survival;
GOG: Gynaecological Oncology Group;
RT: Radiotherapy;
CEA: Carcinoembryonic Antigen;
CA 19.9: Carbohydrate Antigen 19.9;
CA 125: Carbohydrate Antigen 125;
NPV: Negative Predictive Value;
US: Ultrasonography

Vol.2, No.7, 661-671 (2010)
doi:10.4236/health.2010.27100

Health

Computer-assisted anti-AIDS drug development: cyclophilin B against the HIV-1 subtype A V3 loop

Alexander M. Andrianov[1]*, Ivan V. Anishchenko[2]

[1]Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, Minsk, Belarus;
[*]Corresponding Author: andrianov@iboch.bas-net.by
[2]United Institute of Informatics Problems, National Academy of Sciences of Belarus, Minsk, Belarus

Received 16 December 2009; revised 24 March 2010; accepted 26 March 2010.

ABSTRACT

Aim: The objects of this study originated from the experimental observations, whereby the HIV-1 gp120 V3 loop is a high-affinity ligand for immunophilins, and consisted in generating the structural complex of cyclophilin (Cyc) B belonging to immunophilins family with the virus subtype A V3 loop (SA-V3 loop) as well as in specifying the Cyc B segment forming the binding site for V3 synthetic copy of which, on the assumption of keeping the 3D peptide structure in the free state, may present a forward-looking basic structure for anti-AIDS drug development. Methods: To reach the objects of view, molecular docking of the HIV-1 SA-V3 loop structure determined previously with the X-ray conformation of Cyc B was put into practice by Hex 4.5 program (http://www.loria.fr/~ritchied/hex/) and the immunophilin stretch responsible for binding to V3 (Cyc B peptide) was identified followed by examination of its 3D structure and dynamic behavior in the unbound status. To design the Cyc B peptide, the X-ray conformation for the identical site of the native protein was involved in the calculations as a starting model to find its best energy structural variant. The search for this most preferable structure was carried out by consecutive use of the molecular mechanics and simulated annealing methods. The molecular dynamics computations were implemented for the Cyc B peptide by the GROMACS computer package (http://www.gromacs.org/). Results: The overmolecular structure of Cyc B with V3 was built by computer modeling tools and the immunophilin-derived peptide able to mask effectively the structurally invariant V3 segments embracing the functionally crucial amino acids of the HIV-1 gp120 envelope protein was constructed and analyzed. Conclusions: Starting from the joint analysis of the results derived with those of the literature, the generated peptide was suggested to offer a promising basic structure for making a reality of the protein engineering projects aimed at developing the anti-AIDS drugs able to stop the HIV's spread.

Keywords: HIV-1; V3 Loop; Cyclophilin B; Computer Modeling; Molecular Docking; Anti-Aids Drug Design

1. INTRODUCTION

The HIV-1 envelope glycoprotein (Env), the etiologic agent of AIDS [1], consists of two noncovalently bound subunits derived from the gp160 precursor. One of these subunits, gp120 protein, is localized on the surface of the viral isolates and becomes a direct party to the virus binding to the target-cells, whereas the other, transmembrane gp41 protein, triggers the process of membrane fusion resulting in the invasion of the virus genome into the macrophages and T-lymphocytes [2]. Specific interactions of the HIV-1 with the virus primary receptor CD4 as well as with its chemokine co-receptors CCR5 and/or CXCR4 are put into effect using the V1-V5 loops of gp120 disclosing the high variability of the amino acid sequences in diverse virus strains [3-5]. Currently, special emphasis of the research teams involved in the anti-AIDS drug studies is attracted to the HIV-1 V3 loop (reviewed in [6]). The higher interest in V3 is caused by numerous experimental data [7] testifying to the fact that exactly this gp120 site gives rise to the principal target for neutralizing antibodies and accounts for the choice of co-receptor determining the preference of the virus in respect with T-lymphocytes or primary macrophages [8]. The differential usage of co-receptors, which is critically dependent on the sequence, charge, and/or structure of the V3 region of gp120 [9,10], dictates the viral phenotype, which shows a typical pattern of evolution during the natural history of HIV-1 infection. CCR5-restricted strains (R5) are the most prevalent in vivo, as they are almost invariably responseble for the initial transmission, predominate during the long asymptomatic phase of the infection, and often persist after the progression to full-blown AIDS; by contrast, strains that utilize CXCR4, either alone (X4) or in combination with CCR5 (R5X4), emerge only in a subset of patients, typically in conjunction with the onset of clinical signs of disease progression and immune system deterioration [11,12].

Since the V3 loop governs the cell tropism and cell fusion [7], one of the strategic ways in developing the anti-HIV-1 drugs may be based on the approach anticipating the search for the chemicals able to block efficiently this functionally significant stretch of gp120 [6]. Comprehensive analysis of the data given in study [13] allows one to suppose that immunophilins exhibiting specific high-affinity interactions with the HIV-1 V3 loop may be utilized as a basic substance to set out of the search for the potential anti-AIDS therapeutic agents.

Immunophilins known originally as the cellular receptors of the immunosuppressive drugs cyclosporine A and FK506 or rapamycin organize the extensive group of proteins exhibiting peptidyl-prolyl cis-trans isomerase activity which is inhibited specifically and efficiently on binding of the corresponding immunosuppressant [14]. Immunophilins subdivided into three families of proteins, namely cyclophilins and FK506-binding proteins (FK BP_S), and a novel chimeric dual-family immunophilin, named FK506- and Cyclosporine-binding protein (FCBP) show similar enzymatic and biological functions despite the apparent difference in their sequence and three-dimensional structures [15]. Alongside with the function of intracellular receptors of immunosuppressants, individual representatives of immunophilins act as catalysts of protein folding and as shaperones stabilizing proteins in a defined conformation and supervising the quality of their spatial structure [16,17]. A variety of bacterial and protozoan pathogens express FKBP-related peptidyl- prolyl cis-trans isomerases termed macrophage-infectivity potentiators (Mip). Mip proteins act in host cell infection as virulence factors, either as membrane-bound proteins on the surface of the pathogens or as soluble secreted proteins [18,19]. The peptidyl-prolyl cys-trans isomerase activity of Mip proteins is suppressed by FK506, which reduces the infectivity of the pathogens without affecting the rate of intracellular replication. Distinct immunophilins were found to be released from cells. Cyclophilin B was detected in human milk [20] and blood plasma [21], but is mainly localized in the endoplasmatic reticulum of cells. The cytosolic immunophilins cyclophin A and FKBP12 were shown to be released during apoptosis of fibroblasts [22] and to act as chemokines by unknown mechanism [23-25]. Recent researches have revealed that many immunophilins possess a shaperone function independent of peptidyl-prolyl cis-trans isomerase activity (reviewed in [15]). Knockout animal studies have confirmed multiple essential roles of immunophilins in physiology and development consisting in interactions with proteins to guide their proper folding and assembly [15].

Reasoning from the empirical observations, there is a good motive to think that immunophilins present in normal human blood plasma are directly relevant to the HIV-1 replication assisting the virus with getting into macrophages and T-lymphocytes [26]. In particular, cyclophilin A packaged into nascent virus particles by specific binding to the capsid region of the Gag precursor protein at the time of viral assembly [27-29], was found to mediate the HIV-1 attachment to the target cells via heparans followed by the gp120-CD4 interaction [26]. Due to the interaction of immunophilins with the HIV-1 isolates, their role of conformases or docking mediators in the virus life cycle seems to be highly probable, since immunophilin receptors on cell membranes and immunophilin-related virulence factors of pathogens have been identified [13].

This work proceeds with our previous studies [30,31] where two virtual molecules, namely FKBP and Cyc A peptides, presenting the promising anti-HIV-1 pharmacological substances were designed by means of computer modeling based on the analysis of specific interactions of the FK506-binding protein and cyclophilin A with V3.

The object of the present study was to model the structural complex of one more protein from immunophilins superfamily, cyclophilin (Cyc) B, with the HIV-1 subtype A V3 loop (SA-V3 loop) circulating in Eastern Europe including Republic of Belarus and, therefore, offering the target of our special interest, as well as to specify the Cyc B segment forming the binding site for V3, the synthetic copy of which, on the assumption of keeping the 3D peptide structure in the free state, may be considered as a forward-looking applicant for the role of a new antiviral drug.

To this effect, molecular docking of the HIV-1 SA-V3 structure determined previously [32] with the X-ray conformation of Cyc B was put into practice, and the Cyc B stretch responsible for the binding to V3 was identified followed by predicting the most probable 3D structure of this stretch in the unbound state, studying its dynamic behavior, and collating the results obtained with the X-ray data for the corresponding site of Cyc B. Thereupon, the potential energy function was analyzed for the complex of the SA-V3 loop with the Cyc B peptide offering the virtual molecule that imitates the Cyc B segment making a key contribution to the interactions of the native protein with V3. As a matter of record, the designed peptide was shown to be capable of the effecttive masking of the functionally critical and structurally rigid V3 sites, presenting the suitable framework for making a reality of the protein engineering projects utilizing the V3 target for developing the anti-AIDS drugs able to stop the HIV's spread.

2. METHODS

2.1. Molecular Docking Simulations

Molecular docking of the SA-V3 loop [32] with Cyc B (file 1CYN of the Protein Data Bank [33,34]) as well as with the Cyc B peptide was executed by the Hex 4.5 program [35] which presents an interactive molecular graphics package for calculating and displaying feasible docking modes of pairs of protein and DNA molecules and employs the spherical polar Fourier correlations to accelerate the computations. Energy refinement of the generated complexes was performed in the GROMACS package [36] by minimizing their potential energy. To this end, the conjugate gradient method was used for the complex of the native protein with the V3 loop as well as for the overmolecular ensemble of V3 with the Cyc B peptide. At the final point of computations, the structural complexes were subjected to the procedure of simulating annealing carried out during 100 ps time domain at initial and final temperatures equal to 500 and 0 K respecttively.

2.2. Determination of the 3D Static Structure for the Cyc B Peptide and Molecular Dynamics Computations

To design the 3D structure of the Cyc B peptide, the X-ray conformation of the Cyc B site [37] responsible for its binding to V3 was involved in the calculations as a starting model to find its best energy structural variant in the unbound form. The search for this most preferable conformation was executed by consecutive use of the molecular mechanics and simulated annealing methods realized in the Tinker package [38] with activating its program modules Minimize and Anneal.

The molecular dynamics (MD) simulations of the built Cyc B peptide structure were implemented by the GROMACS computer package [36] using the GROMOS96 force field parameter set 53A6 [39]. The starting 3D structure of the CycB peptide generated hereinbefore was placed in a cubic box so that the smallest distance between its walls and the peptide atoms was greater than the half of the cut-off radius of the Coulomb and Lennard-Jones potentials fixed at 1.4 nm. Simple point charge water model [40] was utilized to set the parameters of explicit solvent on which the periodic boundary conditions were imposed in all directions. Before the MD computations, the initial Cyc B peptide model was subjected to the procedure of energy minimization realized in vacuum by the steepest descent method. The MD simulations were carried out at temperature 310 K during 20.5 ns time domain with 1 fs step at fixed pressure and number of atoms, the first 0.5 ns being the stage of solvent relaxation. To integrate the Newton's equations of motion, the common leap-frog algorithm was used. To control the temperature, the weak coupling scheme to an external bath [41] was employed in the calculations with 0.1 ps characteristic time. As with the temperature coupling, the system was linked to a "pressure bath" by exponential relaxation of pressure [41] with 1.0 ps time constant.

Every 10 ps, the geometric parameters of the MD structures and the data on their energy characteristics were recorded into the trajectory file. Comparison of the MD conformations between themselves and with the input structure was performed in terms of the values of root-mean-square deviations computed both in Cartesian and angular space. To this effect, the GROMACS routines [36] were implicated in the studies.

The computations were run in parallel on SKIF K-1000 computer cluster on 64 CPUs [42].

2.3. Identification of Secondary Structures in the Cyc B Peptide

To determine the different types of secondary structures in the Cyc B peptide, the ϕ, ψ values for all of the amino acids derived from the simulated model were analyzed in compliance with the criteria given in study [43]. The types of β- and γ-turns were identified within the classification of Hutchinson and Thornton [44]. To detect the nonstandard β-turns, the additional information on the distances $C^{\alpha}_{i}\ldots C^{\alpha}_{i+3}$ computed from the atomic coordinates of the simulated structures was employed.

2.4. Collation of 3D Static Structures

The values of root-mean-square deviations (RMSD) in atomic coordina tes (cRMSD) were taken to evaluate the similarity of the structures in the Cartesian space [45]. To compare the structures in terms of the dihedrals, the RMSD between corresponding angles (aRMSD) were used as a measure of their conformational similarity in the angular space [45].

3. RESULTS AND DISCUSSION

Figure 1 casts light on the structural complex of the HIV-1 SA-V3 loop with Cyc B generated via molecular docking of their 3D structures followed by optimization of its geometric parameters. Insight into the function describing the energy surface of the built complex makes it clear that the binding of V3 to Cyc B initiates the formation of stable overmolecular structure that is characterized by the value of potential energy equal to –6434 kcal/mol. Analysis of the matrix of interatomic contacts coming true in the designed complex allows one to identify the amino acids of V3 and Cyc B participating in the intermolecular interactions the total energy of which comes to –75 kcal/mol. So, according to the data obtained, such V3 residues as Ser-11, Val-12, Gly-15, Pro-16, Gly-17, Gln-18, Ala-19, Thr-23, and Arg-31 take up positions nearby the surface of Cyc B giving rise to the binding site for V3 by means of Gly-1, Pro-2, Lys-3, Gly-28, Lys-29, Thr-30, Lys-91, Lys-93, and Glu-178. One needs to note that cooperation of the V3 loop with Cyc B results in the origin of one ion pair organized by Arg-31 of V3 and Glu-178 of Cyc B and in the formation of six H-bonds that appear as a result of donor-acceptor interactions of the receptor amino acids Lys-91, Lys-93, and Glu-178 on the one hand as well as of V3 residues Ser-11, Val-12, Gln-18, Ala-19, and Thr-23 on the other hand (see information given in **Table 1**).

These results signify that interaction of the V3 loop with Cyc B entails the blockade of its central region making the immunogenic crown of gp120 [46], whereas the residues of V3 N- and C-terminal segments (except

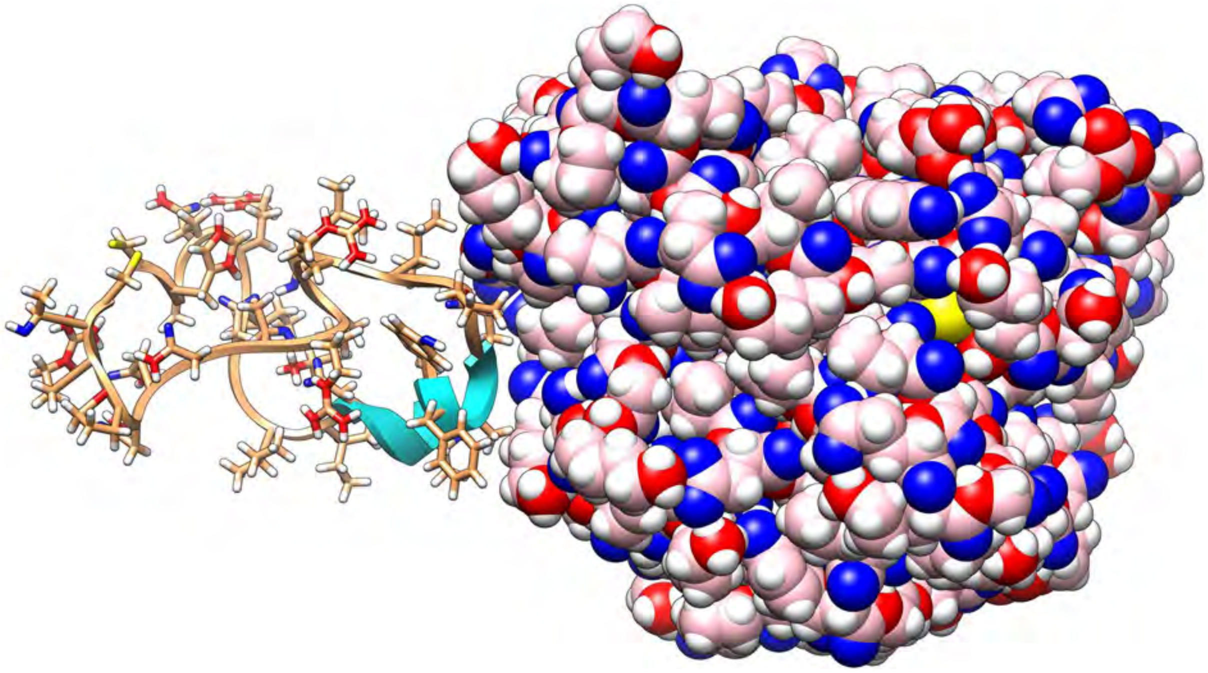


Figure 1. Image of the structural complex between the HIV-1 SA-V3 loop (tubes) and Cyc B (balls).

Table 1. Geometric parameters of intermolecular H-bonds for the structural complex of the HIV-1 SA-V3 loop with Cyc B.

Residue (donor)	Group (donor)	Residue (acceptor)	Group (acceptor)	Distance (Å) Donor…Acceptor	Distance (Å) Hydrogen…Acceptor
Lys-93[2]	NH	Gln-18[1]	OE1	2.7	1.7
Lys-93[2]	NZ	Thr-23[1]	OG1	2.8	1.8
Ser-11[1]	OG	Glu-178[2]	OE2	2.7	1.7
Gln-18[1]	NH	Lys-91[2]	CO	2.8	1.9
Gln-18[1]	NE2	Glu-178[2]	OE2	2.8	1.9
Ala-19[1]	NH	Lys-91[2]	CO	3.0	2.0

Footnote: Superscripts 1 and 2 denote the amino acids of V3 and Cyc B respectively.

for Arg-31) relating to its stem [46] do not come in direct contact with the receptor. The data above are in harmony with those of study [13], whereby high affinity to immunophilins is typical not merely for intact V3 variable loops but also for their peptides embracing the immunogenic tip of gp120. Among the segments of V3 interacting effectively with Cyc B, it is essential to mark its tripeptide Gly-15-Pro-16-Gly-17 occurring actually in all of the deciphered amino acid sequences of the HIV-1 principal neutralizing determinant [47]. Functional role of this invariant V3 stretch has not been completely specified. Nevertheless, it as known that, even a single substitution for its central residue by alanine makes an impact both on the virus immunogenicity and infectivity [48] testifying to important role of Pro-16 in the HIV-1 life cycle. Under the data derived, the 3D structure of the V3 fragment of interest is practically identical to that of Cyc B site Gly-1-Pro-2-Lys-3 which is spatially close to it: the value of cRMSD computed for all of the atoms of their main chains totals 0.7 Å. Resem- blance of the 3D main chain shapes observed for the two segments of the ligand and the receptor makes it possible to suggest that Cyc B stretch Gly-1-Pro-2-Lys-3 gives rise to the signal structure that is interpreted by V3 as a mirror image of its own immunogenic crest, which, most likely, presents the head reason involving the specificity of V3 interactions with immunophilins. In this light, the findings above confirm the validity of the assumption made in our previous studies [30,31] where specific high-affinity interactions of the HIV-1 V3 variable loops with immunophilins arising from experimental observa- tions [13] were suggested to be stipulated by appearing in their amino acid sequences the fragments exposing the similar 3D structures which are constructed from β-turns of polypeptide chain (for details see works [30,31]).

In such a way, the data of molecular docking testify to realizing the energetically favorable contacts of the HIV-1 SA-V3 loop with Cyc B resulting in the masking of some of the key V3 amino acids of its immunogenic crown. In this context, we could suggest of a possible usage of immunophilins and, in particular, Cyc B as an alternative to the V3-directed antibodies commonly used to neutralize the HIV-1 activity. However, the evidence of study [49] demonstrating that increase of immunophilins concentration in infected blood plasma does not influence the virus infectivity conflicts with this primitive conjecture. In the case of Cyc B, the probable cause of its insufficient neutralizing activity may consist in the fact that, as follows from our simulations, the binding of the immunophilin to the HIV-1 V3 loop occurs via interactions with the central region of V3 and does not affect its N- and C-terminals (**Figure 1**) where the major portion of the residues involved in cell tropism and cell fusion is localized [50-52]. Therefore, to amplify the blockade of V3 and preserve its capacity for specific interactions with Cyc B, we have undertaken an attempt to design as potential anti-HIV-1 drug the virtual molecule named Cyc B peptide and imitating N-terminal segment 1-30 of the native immunophilin. The choice of Cyc B segment 1-30 for continuation of our studies is caused by the following motive: in compliance with the designed data, it holds tripeptide Gly-1-Pro-2-Lys-3 recognizable by the virus immunogenic crest and comprises significant share of the residues making the binding site for V3. Certainly, such a definition is correct only in case that the 3D structure of this Cyc B segment does not experience the considerable alterations in its free state. To check whether that is true, we computed the most preferable 3D structure of the Cyc B peptide and compared it with the one appearing in crystal [37] in the corresponding site of the intact protein. Analysis of **Figure 2** illustrating the image of the superposed peptide structures gives grounds to conclude that the spatial folds of their backbone are closely related, and this inference arising from visual observation is ratified by the value of cRMSD equal to 2.4 Å. At collating the structures given in **Figure 2**, it is essential to underscore that very close agreement between them (cRMSD is 0.5 Å) occurs in segment Gly-1-Pro-2-Lys-3 that, as stated above, forms in the native Cyc B the conformational epitope specifically recognizable by V3. Analogous conclusion to the effect that the compared structures are alike follows from their confrontation in the conformational space (ϕ, ψ): the value of aRMSD calculated for all of the peptide amino acids comes to 33°.

Insight into the static model for the 3D structure of the Cyc B peptide (**Figure 3(a)**) shows that essential contribution to its energy stabilization belongs to the donor-acceptor interactions that result in forming the extensive network of hydrogen bonds appearing between amino acids both distant and adjacent in the polypeptide chain. The molecule generated by computer modeling tools offers the elongated “construction” in which the spatially close N- and C-terminal residues give rise to the long-range H-bond by the oxygen of Gly-1 carboxyl group and the hydrogen of hydroxyl group of Thr-30 side chain. As follows from the dihedral values given in **Table 2**, central part 10-22 of the Cyc B peptide constitutes the β-sheet the “oval isthmus” of which is composed from consecutive β-turns (**Figure 3(b)**), with their spatial folds being similar to those previously [32] in the immunogenic crown of the HIV-1 SA-V3 loop. In particular, according to our simulations, tetrapeptide Ile-14-Gly-15-Asp-16-Glu-17 of the Cyc B peptide adopts the conformation of none-standard β-turn IV close to that of stretch Gly-15-Pro-16-Gly-17-Gln-18 of V3: in this case, the

Table 2. Dihedral angles for amino acids in the 3D structure of the Cyc B peptide.

Residue	Dihedral angles (deg.)				
	ϕ	ψ	χ_1	χ_2	χ_3
Gly-1	—	–109.7	—	—	—
Pro-2	–52.2	178.6	–18.1	31.0	
Lys-3	–135.3	170.1	–64.6	–176.2	–69.6
Val-4	–84.6	141.8	176.6	—	—
Thr-5	–135.9	–136.8	69.1	—	—
Val-6	–60.6	123.4	–62.0	—	—
Lys-7	–93.7	80.2	–173.1	59.0	168.0
Val-8	–112.5	160.8	–153.8	—	—
Tyr-9	–78.7	170.2	78.2	–82.8	—
Phe-10	–144.3	161.7	–168.0	–101.0	—
Asp-11	–85.7	165.1	–141.6	–58.4	—
Leu-12	–147.4	101.0	–154.5	–50.8	—
Arg-13	–129.4	102.0	–45.8	–162.4	70.9
Ile-14	–80.3	88.7	–55.0	–179.9	—
Gly-15	74.1	–72.1	—	—	—
Asp-16	–147.8	5.8	–152.3	–61.9	—
Glu-17	–53.7	120.6	–64.8	–176.4	–30.7
Asp-18	–74.2	42.0	–157.7	–137.1	—
Val-19	–52.9	–31.1	–165.5	—	—
Gly-20	118.2	–175.8	—	—	—
Arg-21	–80.9	55.8	–75.7	157.4	–70.7
Val-22	–53.2	129.0	179.8	—	—
Ile-23	–76.6	60.7	–39.7	–59.3	—
Phe-24	–56.9	–63.3	–66.1	–75.9	—
Gly-25	77.9	171.0	—	—	—
Leu-26	–138.0	159.7	–66.5	95.1	—
Phe-27	–97.0	–5.3	–51.8	–83.5	—
Gly-28	61.7	–130.8	—	—	—
Lys-29	–133.8	143.2	176.9	63.7	169.4
Thr-30	–122.6	—	–62.2	—	—

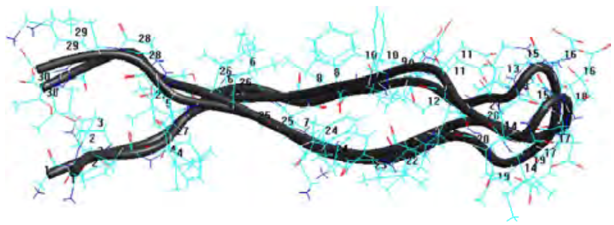
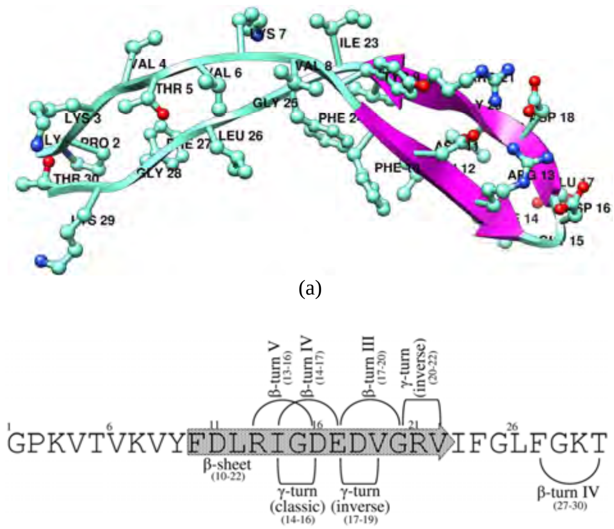


Figure 2. 3D structure of the Cyc B peptide superposed with the X-ray conformation for segment 1-30 of the entire protein.



(a)

(b)

Figure 3. (a) Three-dimensional; (b) structures of the Cyc B peptide generated based on the X-ray conformation of site 1-30 for the intact Cyc B.

value of cRMSD estimated for the backbone atoms of the compared structures is 1.1 Å. Resemblance in the structural organization of the central regions of V3 and the Cyc B peptide takes also place for their longer fragments. For instance, if we compare the 3D structure of V3 stretch 15-20 producing the overwhelming majority of contacts with neutralizing antibodies (study [53]) with the one of the Cyc B peptide segment 14-19, it is also possible to observe the conformity of their spa- tial backbone folds (the corresponding value of cRMSD aggregates 2.0 Å). This outcome enables one to assume that, subject to the observance of the principle of "mirror similarity" formulated in studies [30,31], segment of the Cyc B peptide forming the "oval isthmus" of the β-sheet and located in the native protein inside its globule may give an additional site for specific binding to V3 alternative to stretch Gly-1-Pro-2-Lys-3 of the intact immunophilin.

When looking into the secondary structure of the designed molecule (**Figure 3(b)**), one cannot but catch sight of the peptide segment 27-30 that, as the stretches of its central part, exposes the conformation of β-turn, which merits the principal concern in view of the data on the 3D structure of the SA-V3 loop [32] where the C-terminal site organizes exactly the same structural motif. This evidence combined with that above implicates the following conclusion: the secondary structures of V3 and the Cyc B peptide observed in their central and C-terminal portions are closely related. In addition, considering the main chain dihedrals (**Table 2**) indicates that, except β-turns, the analyzed structure also forms γ-bends the central residues of which are located in positions 15, 18, and 21 (**Figure 3(b)**).

The results of molecular dynamics simulations implemented during 20 ns time domain by applying the static 3D structure of the Cyc B peptide as the starting model are evidence of its relative conformational rigidity: the average of cRMSD calculated for the structures of the MD trajectory and starting point amounts 3.1 Å and the system of intramolecular H-bonds serving as one of the factors stabilizing the molecule structure undergoes no drastic changes within the whole MD trajectory. Nonetheless, comprehensive study on the dynamic structures of Cyc B peptide indicates that their individual representatives differ significantly from the input structure, which does not exclude the probability of wide-ranging structural reorganizations of this molecule stimulated by abrupt alterations of the environment that, for example, may happen upon its entry into numerous intermolecular interactions.

Conformity of the 3D structure of the Cyc B peptide with that of the same immunophilin segment (**Figure 2**) and its relative structural inflexibility following from the data of molecular dynamics computations give ground to believe that the molecule designed here may not only conserve the capacity for specific interaction with V3 characteristic of the native protein [13] but also intensify the blockade of this cryptic site of gp120. Indeed, delving into the potential energy function describing the structural complex of the Cyc B peptide with V3 illustrates (**Figure 4**) that, as compared to the native protein, the peptide originating from its framework exhibits much more extensive network of contacts with the V3 segments embracing the biologically significant amino acids of gp120. So the energy of intermolecular interacttions in the complex of Cyc B with V3 amounts to –75 kcal/mol and, in the case of interest, its value falls down to –350 kcal/mol. And at the same time, stabilization of the complex between the Cyc B peptide and V3 is reached owing to the multiple donor-acceptor interactions (see **Table 3**) as well as to the salt bridge formed using Arg13 of V3 and Asp-18 of immunophilin-derived peptide. When analyzing the system of H-bonds given in **Table 3**, there is need to note that, from the side of V3, contribution to its formation belongs to such biologically meaningful residues of gp120 as Lys-10, Arg-13, Gly-17, Gln-18, Asp-25, Asp-29, Ile-30, and Arg-31 which find themselves to be isolated as a result of arising the overmolecular ensemble. Among the residues of this register, we ought to notice Asp-25 that takes an active part in binding of the virus to the cell membrane surface [54-58] and, along with Ser-11, accounts for the HIV-1 phenotype [59,60]. Constituting the complex of the Cyc B peptide with V3 also entails the masking of its functionally critical amino acids Ser-11, Ala-19, Ile-23, Gly-24, and Gln-32 which are also utilized by the virus to set up the cell tropism determinant [54-58]. The active center of V3 responsible for binding to Cyc B peptide contains Asn-6 the blockade of which may be highly effective for the virus inactivation: as mentioned above, this amino acid of V3 presenting the integral part of its structurally invariant segment 3-7 [32] gives rise to one of the conserved sites of N-linked glycosylation of gp120 [61].

When examining the overmolecular ensemble represented in **Figure 4**, one needs to cast a glance at the following feature: in this complex, segment Gly-1-Pro-2-Lys-3 of the Cyc B peptide interacts with structurally rigid stretch 28-32 of the HIV-1 V3 domain [32], whereas the corresponding site of the native protein, as stated before, contacts the immunogenic crown of gp120 (**Figure 1**). At the same time, this V3 region that proves to be unused by the N-terminal site of the Cyc B peptide becomes very intimate with pentapeptide Ile-14-Gly-15-Asp-16-Glu-17-Asp-18 belonging to the "oval isthmus" of its β-sheet. These observations are of special interest since the indicated segments of the receptor and ligand reside in the β-turns of polypeptide chain which may serve as docking sites for protein-protein interactions [62-64]. The presence of β-turns in the 3D structures of V3 and the Cyc B peptide is likely to be one of the head factors that may make a determinative contribution to the specificity of their efficacious interactions.

Collating the 3D structures of V3 and the Cyc B peptide in natural and constrained states indicates that, in either case, forming the complex brings in the certain structural rearrangements taking place both in the Cartesian and angular spaces. At the same time, the Cyc B peptide experiences the more profound transformation of its structure: so when we confront the V3 structures materialized in the overmolecular ensemble and in the unbound status, the values of cRMSD and aRMSD are respectively 2.0 Å and 47° and, in the case of the Cyc B peptide, the corresponding values rise to 4.0 Å and 57°. This observation falls into line with the supposition above, whereby the Cyc B peptide, in spite of the relative conformational rigidity, may exhibit the higher flexibility of the polypeptide chain on drastic medium alterations.

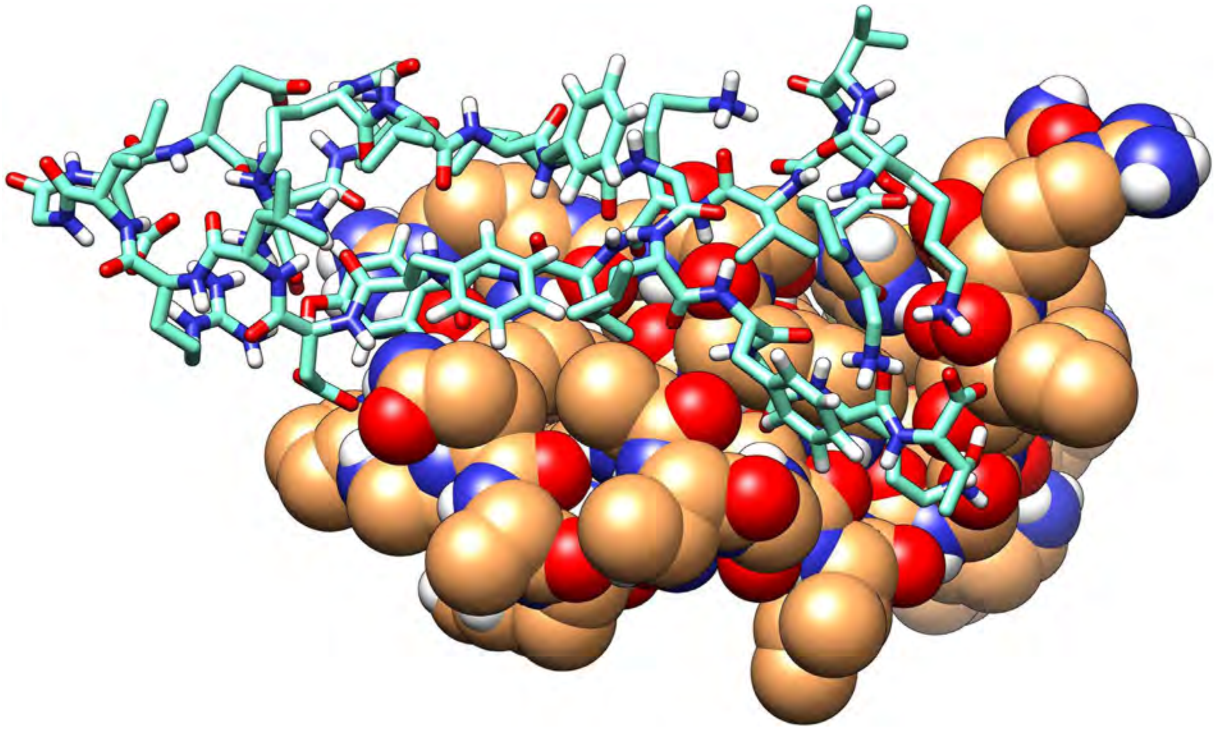


Figure 4. Overmolecular structure of the HIV-1 SA-V3 loop (balls) with the Cyc B peptide (tubes).

Table 3. Geometric parameters of intermolecular H-bonds for the structural complex of the HIV-1 SA-V3 loop with the Cyc B peptide.

Residue (donor)	Group (donor)	Residue (acceptor)	Group (acceptor)	Distance (Å) Donor…Acceptor	Distance (Å) Hydrogen…Acceptor
Arg-13[1]	NH1	Asp-18[2]	OD1	3.0	2.0
Arg-13[1]	NH2	Asp-18[2]	OD1	3.2	2.3
Arg-13[1]	NH2	Asp-18[2]	OD2	3.1	2.2
Gly-17[1]	NH	Asp11[2]	OD1	2.9	1.9
Gln-18[1]	NE2	Tyr-9[2]	CO	2.8	1.8
Gln-18[1]	NE2	Asp-11[2]	OD1	2.8	1.8
Asp-25[1]	NH	Gly-28[2]	CO	2.8	1.8
Lys-3[2]	NZ	Ile-30[1]	CO	2.8	1.9
Thr-5[2]	OG1	Arg-31[1]	CO	2.7	1.7
Lys-7[2]	NH	Lys-10[1]	CO	2.8	1.8
Lys-29[2]	NZ	Asp-29[1]	CO	2.9	2.0

Footnote: Superscripts 1 and 2 denote the amino acids of V3 and the CycB peptide respectively.

4. CONCLUSIONS

In studies [30,31], we implemented the computer-aided design of two molecules referred to as Cyc A and FKBP peptides and, having analyzed their structural complexes with the HIV-1 SA-V3 loop [32], disclosed that the Cyc A peptide binds effectively to its immunogenic crown, whereas the FKBP peptide prefers to interact with the N- and C-terminal segments of the virus principal neutralizing determinant. The findings derived here bear witness that, unlike the molecules constructed previously [30,31], the Cyc B peptide is able to mask the functionally crucial amino acids both of the V3 central part and of its stem stretches. Moreover, as compared to these molecules, cooperation of the Cyc B peptide with V3 brings in the origin of more stable overmolecular structure: for instance, the value of the energy of intermolecular interactions computed for the structural complex of V3 with Cyc A peptide [31] totals -87 kcal/mol and, in the case in question, it aggregates -350 kcal/mol (see above).

As shown in study [32], in spite of the hypervariability of V3, its segments 3-7, 15-20, and 28-32 embracing the highly conserved amino acids of gp120 give rise to the closely related spatial backbone folds in different virus isolates, and, therefore, they may be considered as promising targets for anti-AIDS drug studies. Allowing for these data in common with the evidence which bears witness that the Cyc B peptide is capable of masking the V3 functionally critical residues residing in its structurally invariant segments, one may expect that synthetic copy of this virtual molecule (or its structural analogs) may display biological activity to various HIV-1 strains exhibiting a broadly neutralizing effect. Beyond all shadow of doubt, the peptide constructed here must experience the extensive experimental test to be considered as the coming applicant for the role of "magic bullet" displaying a wide-range blockade of the HIV-1 envelope glycoprotein gp120.

In conclusion, the model of the structural complex of Cyc B with V3 proposed above substantiates the literature data on a high affinity of immunophilins to V3 [13], and the results derived from its analysis enable one to make an optimistic prognosis of the prospects of using their peptides as the starting chemicals for the design of efficacious antiviral agents by protein engineering methods.

5. ACKNOWLEDGEMENTS

This study was supported by grants from the Union State of Russia and Belarus (scientific program SKIF-GRID; № 4U-S/07-111) as well as from the Belarusian Foundation for Fundamental Research (project X08-003).

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Vol.2, No.7, 672-675 (2010)
doi:10.4236/health.2010.27101

Granulomatous interstitial lung disease in a long-term drug abuser

Karen Laudenbach*, Jan Koch, Bernd Seese

Thoraxzentrum Bezirk Unterfranken, Münnerstadt, Germany; *Corresponding Author: K.Laudenbach@tzbu.de

Received 3 March 2010; revised 14 April 2010; accepted 15 April, 2010.

ABSTRACT

It is the habit of some drug consumers to dissolve the powder of crushed pills, intended for oral use, in water and inject this solution intravenously. Insoluble particles than obstruct pulmonary vessels causing microscopic pulmonary emboli. These foreign bodies migrate and penetrate into the perivascular space and interstitium, resulting in chronic inflammation and foreign body giant cell reaction. As a result of this a granulomatous interstitial fibrosis can develop, which has also been described as pulmonary talcosis. We are reporting the case of a 22 year old male with a history of long-term intravenous drug abuse. He presented to our hospital complaining of dyspnoea, cough and generalized weakness. We describe an extensive diagnostic process concluded by an open lung biopsy establishing a definitive diagnosis of this rare granulomatous lung disease. This case underlines the importance of a thorough diagnostic work up and the pathogenic potential of foreign material reaching the lung via blood circulation in amongst the differential diagnoses of interstitial lung diseases, especially occurring in this group of patients.

Keywords: Pulmonary Talcosis; Granulomatous Pneumoconiosis; Drug Abuse; Heroin; Foreign Body Granuloma; Interstitial Lung Disease

1. CASE REPORT

We report of a 22 year old male Patient with a history of intravenous heroine use since he was 15 years old. His general health had been deteriorating for some months. At presentation he complained of increasing shortness of breath on exertion, fatigue and fainting episodes.

2. PHYSICAL EXAMINATION

The patient seemed malnourished and overall in poor health. On auscultation he had normal breath sounds. A systolic murmur over the tricuspid valve area was noted.

Examination of the abdomen, musculoskeletal system and nervous system was without pathological findings.

3. DIAGNOSTIC RESULTS

Chest Radiograph: Showed diffuse interstitial pulmonary nodules.

Blood results: ACE 57 U/l (< 52), CRP 10.2 mg/dl, specific IgG against mould and candida-negative, D-Dimer 1.3 mg/l (< 0.3), BNP 1433pg/ml Serology for Legionella and Mycoplasma-negative Anti-HCV-positive, HIV I and II-negative.

Arterial Blood Gases (ABG): pH: 7.48 $pCO_2$28 mmHg, $pO_2$87 mmHg, Bicarbonate 22.8 mval/l. BE –1, 9.

Pulmonary Function Test: (Bodyplethysmography) Forced expiratory volume (FEV1) 2.21 (53% predicted), vital capacity (VC) 2.821 (56%), FEV1/VC 78% (94%), Total lung capacity (TLC) 4.671 (74%), Gas transfer (TLCO) 5.24 mmol/min/kPas (47%).

Echocardiography: Tricuspid regurgitation (Grade 3), systolic pulmonary artery pressure (PAPsyst.) 37 mmHg + central venous pressure.

Electrocardiography: Sinusrhythm, Right bundle branch block

High-resolution-CT Thorax: diffuse interstitial and fine micronodular infiltration in throughout both lungs (**Figure 1**).

Bronchoscopy: no abnormal findings. Bronchial aspirate: scanty E.coli and Staphylococcus aureus. No acid fast bacilli.

Bronchoalveolar lavage: some smoker macrophages, otherwise unremarkable

Transbronchial biopsy: Evidence of extensive granulomatous interstitial fibrosis.

Under polarized light: granulomas containing crystalline birefringent material (**Figure 2**).

The patients overall condition improved with symptomatic treatments. Over the following month he did however continue to inject heroin. He also admitted to inject a mixture of cigarette ashes and water into his femoral vein on at least one occasion.

Six months later he was re-hospitalized due to right sided chest pain, combined with acute dyspnoea, general weakness and cough with brownish expectoration. He was hypotensive (blood pressure 70/40 mmHg) with a heart rate of 120 bpm.

4. FINDINGS

Blood results: CRP 8.6 mg/dl, D-Dimer 3.6 mg/l, E.coli on sputum examination

Pulmonary function test (Bodyplethysmography): FEV1 1.04l (25% predicted), FEV1/VC 69% (83%), VC 1.49l (30%), TLC 4.37l (69%).

ABG: pH 7.48 pCO_2 23.7 mmHg pO_2 54.4 mmHg

Figure 1. High resolution CT Thorax: diffuse interstitial micronodular pattern with conglomerate formation.

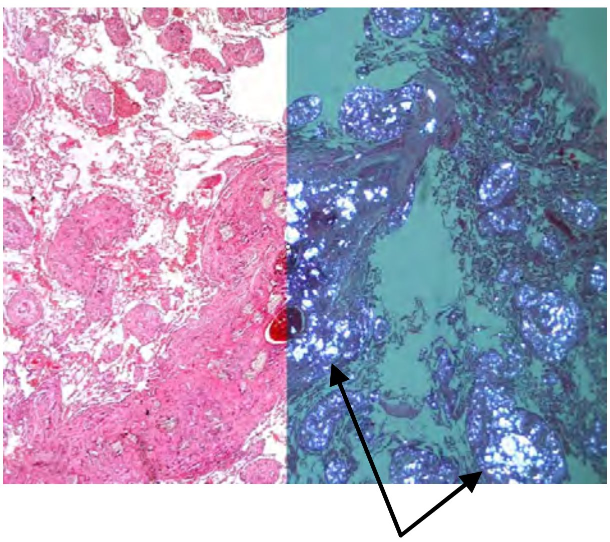

Figure 2. left: H&E-stain, right: under polarized light, positive birefringent material (2.5 × magnified).

Bicarbonate 17.5 mmol/l, BE –4, 4.

High resolution CT Thorax: progressive diffuse interstitial, micronodular changes throughout both lungs. Bilateral small single nodules, one in the right lower lobe measuring 13 mm, one right paracardial measuring 10 mm.

Ventilation/Perfusion scintigraphy 12 days after hospitalization: no evidence of new or old pulmonary emboli.

Doppler ultrasound of the legs: no pathological findings.

Under symptomatic treatment including antibiotics according to sensitivities and oxygen-therapy his condition and cardiopulmonary status stabilized within a few days. Blood gas analysis returned to normal. A short course of systemic Corticosteroids (Prednisolon at a dose of 0.5 mg/kg bodyweight) was given.

In order to establish a definitive diagnosis the patient underwent an open lung biopsy a month later. Tissue sample were obtained from the right lower lobe (S6/9) and the right upper lobe.

Macroscopically the lung had a fine granular appearance. The cut surface presented small up to 4 mm sized brown nodules and a tumourous conglomerate in the specimen from left lower lobe.

Histologically there was strong granulomatous inflammation caused by foreign material with surrounding interstitial fibrosis. The appearances were in keeping with a pulmonary talcosis (drug abuser lung). The specimen from the upper lobe showed a patchy pneumonia with multiple foci of abscess formation.

5. DISCUSSION

Chest radiography and high resolution CT showed a diffuse interstitial lung disease. The pulmonary function test demonstrated moderately severe restrictive and obstructive airflow and a reduction in diffusion capacity.

Patchy pneumonic changes seen in the histological specimen were felt to be of a secondary nature. A primary bacterial or tuberculous origin of the manifest interstitial changes could be excluded. There was nothing in the patient's history to suggest extrinsic allergic alveolitis. Serology for that purpose was also negative.

Transbronchial biopsy as well as open lung biopsy demonstrated histological evidence of granulomatous inflamemation surrounding typical foreign body giant cells. In the centre of these granulomas parts of blood vessels and capillaries could be seen suggesting that foreign material reached the lung via venous circulation. (**Figure 4**) This histological finding is typical for an intravenous pulmonary talcosis (drug abuser lung).

Drug users often inject the powder of crushed pills, intended for oral use, dissolved in water. These often contain a particular filler such as talc, starch or cellulose, magnesium stearate and silica [1,2]. Insoluble particles obstruct pulmonary arteries and capillaries causing microscopic pulmonary emboli. In the following stage these foreign bodies migrate and penetrate into the perivascular space and interstitium where they cause chronic inflammation and provoke a foreign body giant cell reaction [3]. The result is a granulomatous interstitial fibrosis (drug abuser lung). The physician is confronted with a broad clinical spectrum ranging from no symptoms up to fulminant disease and death. Common symptoms are cough, progressive dyspnoea and weight loss [4].

Auscultation may be unremarkable or reveal discrete bibasal crackles.

Sometimes patients present with mild or moderate hypoxia. Pulmonary function tests can be restrictive or obstructive. A typical diagnostic finding is an impairment of gas transfer [5].

Chest x-ray may be normal, but might show compact diffuse masses [6]. Transbronchial biopsy or surgical lung biopsy will confirm the diagnosis of pulmonary talcosis. The characteristic histological finding would be a granulomatous inflammatory reaction, with foreign material enclosed in typical foreign body giant cells (**Figure 3**).

Following long term drug abuse there are a range of patterns in which talcosis can be seen on CT. They consist of fine micronodular pattern, conglomerate parahilar masses on a background of micronodularity, ground glass attenuation, and panacinar or centrilobular emphysema. These patterns frequently appear in combination [7].

Other manifestations include pneumothorax, pulmonary hypertension, pulmonary fibrosis and chronic respiratory failure in the final stage [3]. Up to 80% of long term drug abusers can develop a talc retinopathy. Over 23% of intravenous drug addicts show evidence of septic emboli [8].

Silicosis and sarcoidosis are the two most important differential diagnoses to be considered. Due to the possibility of a coexisting HIV infection miliary tuberculosis should also be thought of and excluded by bronchial lavage.

Miliary metastases and opportunistic respiratory infections such as Pneumocystis jirovecii, CMV or atypical pneumonia (Legionella and Chlamydia) are further important differential diagnoses. An extrinsic allergic alveolitis should be excluded by serology and history taking.

Reports on the natural course and treatment of talc granulomatosis are scarce. Pare *et al.* described irreversible progression of radiographic abnormalities, even after long term abstinence [3]. The most important therapeutic intervention however is to stop any further intravenous drug use, smoking cessation and adequate treatment of significant pulmonary hypertension.

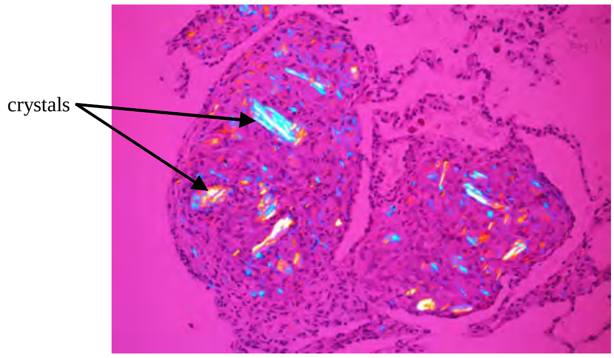

Figure 3. Foreign body granuloma with crystals (10 × magnified).

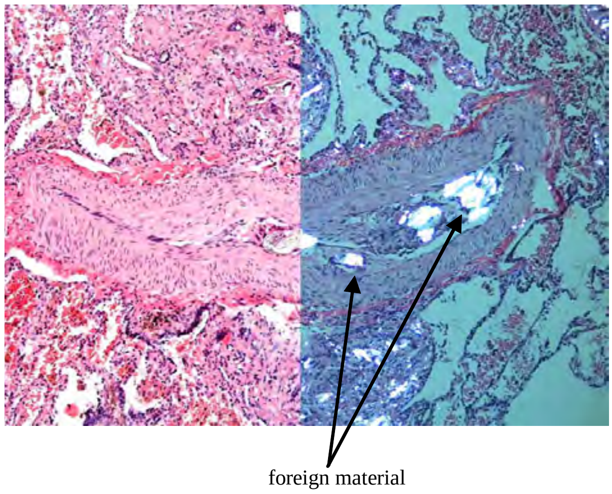

Figure 4. Pulmonary vessel with intraluminal foreign material.

Smith *et al.* describe a positive response to treatment with corticosteroids, initially at a dose of 60 mg Prednisolone daily [4]. In some severe cases of talcosis lung transplantation could be considered, providing complete drug abstinence can be confirmed [9].

6. CONCLUSIONS

Interstitial lung disease needs thorough and often extensive diagnostic clarification.

Whilst inhaled environmental and industrial agents represent an important factor in the pathogenesis of this disease group, we must also consider substances and foreign material reaching the lung via blood circulation and other pathways as described in this case.

It illustrates very well the importance of completing the diagnostic process, reminding the physician of this rare cause of granulomatous lung disease in a specific high risk population.

7. ACKNOWLEDGEMENTS

There is no conflict of interest. The corresponding author confirms, there is no liability to any of the firms mentioned in this article. The presentation is independent and the content remains neutral.

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Vol.2, No.7, 676-681 (2010)
doi:10.4236/health.2010.27102

Health

The association of circulating interleukin-18 with fasting insulin and weight loss in obese children

Tim K. Tso[1]*, Wen-Nan Huang[2], Chen-Kang Chang[3]

[1]Department of Food Science, National Chiayi University, 300 University Road, Chia-Yi, Taiwan, China;
*Corresponding Author: timtso@mail.ncyu.edu.tw
[2]Department of Allergy, Immunology and Rheumatology, Taichung Veterans General Hospital, Taichung, Taiwan, China
[3]Sport Science Research Center, National Taiwan Sport University, Taichung, Taiwan, China

Received 20 February 2010; revised 23 March 2010; accepted 25 March 2010.

ABSTRACT

Obesity is an independent risk factor for developing cardiovascular disease and increases insulin resistance in children. Interleukin (IL)-18 is a novel pro-inflammatory cytokine with potential atherogenetic properties. This study aimed to identify circulating levels of IL-18 in obese children and examine the effects of combined nutritional education-physical activity course on circulating IL-18. Plasma IL-18, body mass index (BMI), fasting glucose and insulin, homeostasis model assessment insulin resistance (HOMA IR), lipid profile, uric acid, high-sensitive C-reactive protein (hs-CRP), and homocysteine were determined in 70 obese children aged 10-12 years before and after attending a 13-week weight reduction program, which included physical activities and nutritional education. Twenty-five age-matched non-obese children served as controls. At baseline, obese children had significantly higher levels of BMI, fasting insulin, HOMA IR, triglyceride (TG), uric acid, hs-CRP, and IL-18 but lower high-density lipoprotein-cholesterol (HDL-C) than non-obese children. Plasma IL-18 levels in obese children decreased significantly after the weight reduction program. At baseline, plasma IL-18 levels in obese children positively correlated with BMI, HOMA IR, insulin and TG but negatively correlated with HDL-C. There was a significant relationship between plasma IL-18 and BMI changes. Moreover, fasting insulin was responsible for IL-18 variability in obese children. These findings suggest that elevated plasma IL-18 levels in obese children are partly associated with parameters of obesity and insulin resistance, and are significantly affected by modest weight loss.

Keywords: Body Mass Index; Children; Insulin; Interleukin; Obesity; Weight Loss

1. INTRODUCTION

The increasing prevalence of childhood obesity has significant medical and economical consequences. Obesity in the youth increases the risk for cardiovascular complications [1]. While mechanisms responsible for the increased prevalence of childhood obesity and associated chronic diseases have not been completely elucidated, its prevention and treatment play an important role in reducing cardiovascular risk. Weight loss induces decreased lipid profiles [2], insulin resistance [3], and chronic inflammatory markers [4] in obese children.

Differential low-grade inflammation is associated with obesity in the youth and some patterns of immune activation are related to insulin resistance [5]. Interleukin (IL)-18 is a novel pro-inflammatory T helper-1 cytokine produced by various cell types, including Kupffer cells, activated macrophages, keratinocytes, intestinal epithelial cells, osteoblasts, and adrenal cortex cells [6]. Recent reports indicate that IL-18 concentrations may be linked to type 2 diabetes mellitus [7,8], metabolic syndrome [9], hyperhomocysteinemia [7,10], obesity [11,12] and insulin resistance [12]. Weight loss and exercise training are reported to reduce IL-18 in obese adults [11,13-15]. However, the association of IL-18 with childhood obesity has not been completely established.

The purpose of the present study was to identify circulating levels of IL-18 in obese children and examine the effects of weight reduction on circulating IL-18.

2. MATERIALS AND METHODS

2.1. Subjects

Seventy 4^{th}-to-6^{th} grade obese Chinese children (37 boys and 33 girls) aged 10-12 years from the local elementary schools were evaluated for plasma IL-18 levels and its relationship to fasting insulin and weight loss before and after completing a 13-week weight reduction program. Obesity was based on the ideal body weight (IBW) criteria and the study children were defined as obese if their body weight was greater than 120% of IBW within age- and gender-specific categories [16]. Twenty-five height- and age-matched children (13 boys and 12 girls) with body weight less than 110% of IBW from the same schools served as non-obese controls.

All of the children had similar lifestyles with no previous physical training program or dietary consults. The ethical committee of the National Taiwan Sport University (Taichung, Taiwan) approved the study and the parents of all participating children provided written informed consent.

2.2. Research Design

The 70 obese children participated in a 13-week weight reduction program consisting of nutritional education and physical exercise programs. Both programs were directed and supervised by dieticians, nutritionists, physicians, physical education instructors, and coaches. Both the children and their parents attended the first six lectures of the nutrition intervention program (45 minutes per lecture, two lectures per week), and the children continued the lectures thereafter.

The nutrition intervention program was taught by school dieticians and was devoted primarily to nutritional education. It included six topics: 1) introduction to the food pyramid, food categories, food and beverage choices; 2) introduction to nutrients and recommended daily nutrient intake; 3) understanding food preparation, balanced diet, eating habits, and over-eating control; 4) learning about dietary recall, food records, and food frequency questionnaire; 5) learning about simple concepts of the food exchange list and practice of calorie calculation; and 6) application and practice of nutrition knowledge by playing games and giving tests.

Regarding physical activity intervention, all obese children were instructed to participate in physical activeity three times per week (45 minutes per exercise session) during the study period supervised by professional youth coaches at the elementary schools. The physical activeties throughout this exercise intervention varied in intensity and duration, but were similar to the type of exercise that elementary school children regularly performed.

2.3. Experimental Approaches

Blood specimens before (baseline) and after (week 13) the 13-week weight reduction program were collected in the morning after an overnight fast and centrifuged immediately to obtain serum or plasma aliquots that were either used immediately or frozen at -80°C until the required assay.

Fasting glucose concentration was determined using an enzymatic colorimetric method (Sigma Chemical Company, St. Louis, MO, USA), while quantitative measurement of fasting insulin concentrations was conducted using an Abbott IMx Insulin Kit based on a micro-particle enzyme immuno-assay (MEIA) (Abbott Laboratories, Dainabot, Tokyo, Japan). HOMA IR was calculated according to the formulas in the HOMA model [17]. Enzymatic methods were used to determine plasma concentrations of total cholesterol (Beckman TC Reagent) and TG (Beckman TG Reagent). Magnesium-dextran sulfate precipitation reagent was used to separate HDL-C, which was then assessed enzymatically. Low-density lipoprotein-cholesterol (LDL-C) was determined using the Friedewald equation [18].

Plasma homocysteine levels were measured using high-performance liquid chromatography with fluorescence detection. Quantitative determination of plasma hs-CRP was assessed by particle-enhanced immunonephelometry using a high sensitivity CRP immunonephelometric assay kit (Dade Behring, Marburg, Germany). Plasma IL-18 levels were assessed by enzyme-linked immunosorbent assay (ELISA) using a Human IL-18 ELISA kit (Medical & Biological Laboratories Co., LTD., Nagoya, Japan) based on the manufacturer's instructions and analyzed with a Dynex MRX II micro-plate reader (DYNEX Technologies, Inc., Chantilly, VA, USA) at a wavelength of 450 nm. This commercial kit was used for detecting only bioactive IL-18, not the immature pro-IL-18, as has been widely used. The intra- and inter-assay coefficients of variation for IL-18 measurements were 7.26% and 7.53%, respectively.

2.4. Statistical Analysis

Tested variables for comparison of means were expressed as mean ± standard error of mean (SEM). The distribution of tested variables was examined graphically for normality. An independent sample t test or Mann-Whitney U test was used to examine mean differences between the obese and the non-obese children. Paired t test was used to compare values obtained before and after attending the 13-week weight reduction program in all obese children. Analysis of variance (ANOVA) was used to compare values obtained from obese children stratified by BMI and fasting insulin levels. Pearson's correlation or Spearman correlation analysis was used to examine the relationships between IL-18 and tested variables. A stepwise multiple regression analysis was then performed to determine independent variables for plasma levels of IL-18. A p value < 0.05 was considered statistically significant. All statistical analyses were performed using the Statistical Package of Social Sciences (SPSS) 10.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. RESULTS

The 70 obese children (37 boys and 33 girls) had an average age of 11 years. All completed the weight reduction program without any reported discomfort or complaints. About 67% (47/70) of them lost weight (mean: –1.69 ± 0.31 kg, range: 0.2-11 kg) and 87% (61/70) had reduced BMI (mean: –1.26 ± 0.13 kg/m^2, range: 0.04-5.62 kg/m^2). In addition to reducing BMI (mean: 27.47 ± 0.38 vs. 26.59 ± 0.40 kg/m^2, $p < 0.001$), the weight reduction program also reduced plasma levels of uric acid (mean: 6.16 ± 0.16 vs. 5.76 ± 0.15 mg/dL, $p = 0.005$), homocysteine (mean: 11.48 ± 0.32 vs. 10.06 ± 0.28 µmol/L, $p < 0.001$), hs-CRP (mean: 1.18 ± 0.02 vs. 1.01 ± 0.02 mg/L, $p < 0.001$), and IL-18 (mean: 161.44 ± 4.03 vs. 132.57 ± 1.48 pg/mL, $p < 0.001$) without significant changes in lipid profile or insulin resistance. There were no gender or age differences for weight loss and BMI reduction (data not shown).

The comparisons of test variables for the 70 obese and 25 non-obese children were shown in **Table 1**. Compared to non-obese children, obese children had lower HDL-C but higher levels of fasting insulin, HOMA-IR, TG, uric acid, hs-CRP, and IL-18. The plasma IL-18 levels of obese children by BMI and fasting insulin tertiles at baseline, and the effects of weight reduction were shown in **Table 2**. There was an increase in plasma IL-18 levels in obese children with higher BMI and fasting insulin. Obese children with fasting insulin in the middle and top tertiles had significantly higher plasma IL-18 levels than those in the bottom tertile. In addition, plasma IL-18 levels of obese children decreased significantly after the weight reduction program.

Table 3 showed the association of plasma IL-18 levels with anthropometric measures and metabolic variables in obese children at baseline. Plasma IL-18 levels in obese children at baseline positively correlated with BMI, insulin, HOMA-IR, and TG but negatively correlated with HDL-C. Moreover, there was a significant relationship between plasma IL-18 and BMI changes (r = 0.352, $p = 0.004$) but no significant correlations between plasma IL-18 changes and those of other tested variables after the weight reduction program (data not shown).

Moreover, stepwise multiple regression analysis was performed with plasma IL-18 as dependent variable and independent variables that included weight, BMI, fasting insulin, fasting glucose, total cholesterol, TG, LDL-C, HDL-C, uric acid, homocysteine, and hs-CRP. The results showed that fasting insulin was responsible for plasma IL-18 variability in obese children (**Table 4**).

4. DISCUSSION

The present study investigated circulating levels of IL-18 in 70 obese children and evaluated the beneficial effects of combined nutritional education-physical activity intervention. The major finding was that obese children have increased plasma IL-18 level, which is associated with BMI and markers of insulin resistance, and is improved with only modest weight loss even without improvement in insulin resistance.

Table 1. Characteristics of the study children[a].

	Non-obese children (n = 25)	Obese children (n = 70)
Gender (boy/girl)	13/12	37/33
Height (cm)	147 ± 1	149 ± 1
Weight (kg)	43 ± 1	61 ± 1*
BMI (kg/m^2)	20.02 ± 0.12	27.47 ± 0.38*
Glucose (mmol/L)	4.26 ± 0.07	4.28 ± 0.06
Insulin (µU/mL)	6.51 ± 0.33	20.28 ± 1.57*
HOMA IR	1.24 ± 0.07	3.91 ± 0.34*
TC (mmol/L)	4.16 ± 0.13	4.17 ± 0.09
TG (mmol/L)	0.65 ± 0.04	0.88 ± 0.05*
LDL-C (mmol/L)	2.38 ± 0.10	2.63 ± 0.08
HDL-C (mmol/L)	1.48 ± 0.05	1.14 ± 0.02*
Uric acid (mg/dL)	4.86 ± 0.25	6.16 ± 0.16*
Homocysteine (µmol/L)	10.88 ± 0.46	11.48 ± 0.32
hs-CRP (mg/L)	0.62 ± 0.06	1.18 ± 0.02*
IL-18 (pg/mL)	95 ± 4	161 ± 4*

Abbreviations: BMI, body mass index; HOMA IR, homeostasis model assessment insulin resistance; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high sensitive C-relative protein; IL, interleukin.

[a]Values are expressed as mean ± standard error of mean.

*$p < 0.05$ compared with non-obese children.

Table 2. Plasma concentrations of IL-18 (pg/mL) by body mass index and fasting insulin tertiles at baseline, and effect of weight reduction in obese children[a].

	Mean ± SEM (95% CI)	P[b]
Body mass index (kg/m^2) tertiles		
< 25.6	157 ± 7 (141-172)	
25.6-28.4	173 ± 7 (159-187)	
> 28.4	172 ± 6 (159-185)	
		0.169
Fasting insulin (μU/mL) tertiles		
< 14.8	151 ± 6 (138-163)	
14.8-21.8	176 ± 9 (158-194)	
> 21.8	176 ± 4 (168-184)	
		0.008
Weight reduction program		
Before	161 ± 4 (114-230)	
After	133 ± 1 (117-158)	
		< 0.001

[a]Values are expressed as mean ± standard error of mean and 95% confidence interval.
[b]Statistical significance ($p < 0.05$) of mean was determined by analysis of variance for body mass index and fasting insulin tertiles, and by paired t test for effect of weight reduction.

Childhood obesity is a great concern because it is associated with many risk factors for chronic diseases later in life. Weight and BMI during childhood have been correlated positively with fasting insulin, blood pressure, and lipids in young adulthood [19]. BMI at age 13 years is highly correlated with that at age 22 years [20]. In addition, subjects with higher BMI as children show higher total cholesterol and LDL-C and insulin resistance in young adulthood [20]. The present study has found that obese children have cardiovascular risk profile in terms of lower HDL-C but higher levels of fasting insulin, HOMA-IR, TG, uric acid, hs-CRP, and IL-18 than non-obese children. After a 13-week combined nutritional education-physical activity program, 67% of obese children had lower body weight with an average weight reduction of 1.69 kg but range that varied from 0.2 to 11 kg. Even though weight loss was limited, the weight reduction program not only reduced BMI but also lowered plasma IL-18 levels as well as other cardiovascular risk factors, such as uric acid, homocysteine, and hs-CRP.

Table 3. Correlation coefficients of IL-18 with anthropometric measures and metabolic variables in obese children at baseline.

	Obese children	
	r	P
BMI	0.378	0.002*
Glucose	–0.183	0.142
Insulin	0.323	0.008*
HOMA IR	0.248	0.044*
TC	0.125	0.316
TG	0.293	0.017*
LDL-C	0.156	0.210
HDL-C	–0.291	0.018*
Uric acid	0.199	0.109
Homocysteine	–0.022	0.858
hs-CRP	0.111	0.374

Abbreviations: BMI, body mass index; HOMA IR, homeostasis model assessment insulin resistance; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high sensitive C-relative protein; IL, interleukin.
*$p < 0.05$ indicated the statistical significance.

Table 4. Stepwise multiple regression analysis in obese children at baseline[a].

	Beta	t value	P value
$R^2 = 0.143$ Adjusted $R^2 = 0.115$ $F = 5.243$			
Fasting insulin	0.287	2.394	0.008

[a]Stepwise multiple regression analysis with plasma IL-18 as dependent variable, while independent variables included weight, BMI, fasting insulin, fasting glucose, total cholesterol, triglyceride, LDL-C, HDL-C, uric acid, homocysteine, and hs-CRP.

Very few studies show an association between IL-18 and obesity in the youth, and obese children with metabolic syndrome have significantly higher IL-18 [21]. IL-18 levels are associated with BMI and waist circumference, fasting insulin, and HOMA-IR in adolescents [5]. In the present study, obese children have significantly higher IL-18 and such an elevation correlates with cardiovascular risk factors such as BMI, HOMA-IR, fasting insulin, TG, and HDL-C (inversely). In addition, fasting insulin explains IL-18 variability in obese children.

There are no reports that show the effects of weight loss on plasma IL-18 in obese children, but an elevation of plasma IL-18 in obese adults can be influenced by interventions related to weight control. Plasma IL-18 decreases significantly after a 12-month weight loss program with energy restrictions in obese women [11]. A 15-week hypo-caloric diet and daily exercise program reduces body weight, plasma IL-18, and increases insulin sensitivity in obese adults [22], while massive weight loss induced by gastric bypass reduces plasma IL-18 [14]. A multi-disciplinary weight reduction program emphasizing lifestyle changes is associated with reduced IL-18 in obese pre-menopausal women [23]. An 8-week exercise training also reduces adipose tissue IL-18 mRNA content in obese adults [15]. In the present study, a 13-week combined nutritional education-physical activity program has reduced plasma IL-18 without significant changes in insulin sensitivity in obese children. After the weight reduction program, 87% of obese children in the present study have shown reduced BMI, but only 41% show decreased HOMA IR and about 43% of BMI-reduced obese children show decreased HOMA IR. Taken together, a decrease in plasma IL-18 caused by the weight reduction program in obese children is at least, in part, due to changes of BMI per se, regardless of the status of insulin resistance.

Systemic inflammation has been demonstrated in overweight children as indicated by elevated CRP levels [24]. It has also been shown that obese children have higher hs-CRP levels than non-obese children [4,25] and weight loss is associated with a significant decrease in hs-CRP levels [4]. Homocysteine concentration is significantly higher in hyper-insulinemic obese children than in the normo-insulinemic obese children [25]. Homocysteine levels are significantly lower after a 12-week weight loss program with dietary advice and light exercise in obese middle-aged women with high homocysteine levels at baseline [26]. In the present study, plasma levels of hs-CRP and homocysteine in obese children have decreased significantly after the weight reduction program. However, there is no relationship among hs-CRP, homocysteine, and IL-18 before and after the weight reduction program. The mechanism associated with elevated circulating IL-18 and the effect of weight loss on IL-18 in obese children seems to be independent from other cardiovascular risk factors, such as CRP and homocysteine, and warrants further elucidation.

The strength of the present study includes the effects of the combined nutritional education-physical activity intervention on obesity-related and/or obesity-independent parameters in obese children, particularly IL-18, which has been better studied in obese adults. However, there are some weakness and limitations. Individual or synergistic effects of nutritional education and exercise on weight changes are not distinguished. In addition, there is no full quantification of the interventions accomplished by the subjects due to lack of dietary records, calorie counts, and estimates of physical activity intensity. Thus, the cause and effect in the relationship between weight loss and improvements of tested variables remain to be elucidated.

In conclusion, there are elevated circulating IL-18 levels in obese children. Modest weight loss through weight reduction intervention has the beneficial effect of reducing IL-18.

5. ACKNOWLEDGEMENTS

This study was supported by a research grant from the National Science Council of Taiwan (NSC 95-2413-H-415-015). The authors wish to thank Ms Zih-Chun Lin for her technical assistance.

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Vol.2, No.7, 682-684 (2010)
doi:10.4236/health.2010.27103

Health

Association of the CLC-Kb T481S polymorphism with childhood hypertension

Rainer Büscher

University of Duisburg-Essen, Children's Hospital, Pediatrics II, Essen, Germany;
rainer.buescher@uk-essen.de

Received 17 March 2010; revised 29 March 2010; accepted 31 March 2010.

ABSTRACT

Essential hypertension is a difficult diagnosis in children and the gene of the renal-epithelial chloride channel *ClC-Kb* is potentially predisposing. *In vitro* studies have shown that a common *ClC-Kb* threonine481serine (T481S) polymorphism leads to enhanced chloride channel activity and may predispose for hypertension (HT). We therefore analysed children at risk for HT for the T481S polymorphism and associated genotype with blood pressure (BP) status. A total of 48 children with essential hypertension (mean age 14.4 ± 2.7 years, 26 male; 22 female; mean BP 143.4 ± 7.5/88 ± 5.8 mmHg) were compared with 78 children with white-coat HT (WCHT), who showed occasionally hypertensive BP values, which were not confirmed by ambulatory blood pressure monitoring (mean age 13.7 ± 2.5 years, 49 male, 29 female; mean BP 122.4 ± 4.3/68.2 ± 3.5 mmHg). Other causes of HT were excluded. Allelic frequencies of hypertensive patients were not significantly different from those with WCHT (HT: A 0.84; T 0.16 vs. WCHT: A 0.85; T 0.15). However, the T-allele was observed more frequently in WCHT subjects with systolic and diastolic BP exceeding the 90th percentile (A 0.71; T 0.29, n = 34, $p < 0.05$, considered as borderline hypertensive). The preliminary data suggest that children with WCHT carry the *ClC-Kb* T481S polymorphism more often and that this variant may predispose for development of arterial HT.

Keywords: Essential Hypertension; White-Coat Hypertension; Ambulatory Blood Pressure Monitoring (Abpm); Clc-Kb T481s Polymorphism; Renal-Epithelial Chloride Channel

1. INTRODUCTION

Essential hypertension in childhood is rare and in most cases, diagnosis can only be confirmed by exclusion of other causes of hypertension (HT) such as renovascular disease, endocrine or cardiovascular disorders [1,2]. In childhood, HT is defined as blood pressure (BP) exceeding the 95th percentile for healthy children and Ambulatory Blood Pressure Monitoring (ABPM) is an important method for the detection and therapeutic monitoring of children and adolescents with HT [3-5]. White Coat Hypertension (WCHT) is the most common diagnosis in children referred for evaluation of HT (> 50%) but there is doubt whether occasionally occurring hypertensive episodes predispose for end-organ damage as adult patient [6].

There is increasing evidence that disturbances in the renal tubular sodium chloride (NaCl) reabsorption are predisposing for the development of HT. The renal-epithelial chloride channel ClC-Kb is expressed in the baso-lateral cell membrane of the distal nephron and is involved in NaCl reabsorption, therefore, the *ClC-Kb* gene might influence development of HT. Recently, a frequent polymorphism has been detected in the *ClC-Kb* gene (threonine to serine amino acid change, T481S) and *in vitro* data confirmed that the *ClC-Kb* T481S polymorphism leads to enhanced activation of the chloride channel [7,8]. Carriers of the *ClC-Kb* T481S polymorphism might therefore be at risk for elevated BP. However, this hypothesis has not been verified in a cohort of hypertensive adult or paediatric patients so far.

The aim of our study is to genotype children and adolescents at risk for development of essential hypertension for the *ClC-Kb* T481S polymorphism and associate the presence of the polymorphism with elevated BP.

2. MATERIALS AND METHODS

Over the past 15 years ABPM has been performed in more than 3000 children in our department. Among those, 126 Caucasian patients, who were seen at least three times in our clinic, were diagnosed as WCHT. ABPM (SpaceLabs 90217 oscillometric device, Redmond, Washington, USA) confirmed hypertensive BP values according to the percentiles of Soergel *et al.* [3] in only 48 patients, while diagnosis was not confirmed in 78 patients. Organic reasons for HT like renovascular or cardiovascular pathology or endocrine disorders were excluded and extended diagnostic analysis did not reveal other feasible reasons for HT in 48 children. All 126 patients (48 HT, 22 female; 78 WCHT, 29 female) were included in our study and EDTA blood samples or buccal swabs were taken during a routine visit in order to generate genomic DNA. Patients were not significantly different regarding age (HT 14.4 ± 2.7 vs. WCHT 13.7 ± 2.5 years), height (HT 153.6 ± 22.6 vs. WCHT 151.5 ± 25.7 cm), weight (HT 57.1 ± 20.4 kg vs. WCHT 56.5 ± 23.9 kg) and BMI (HT 23.17 ± 5.0 vs. WCHT 23.75 ± 5.4 kg/m^2).

Genotyping for the *ClC-Kb* T481S polymorphism was performed using SSCP and direct sequencing as described before [7,8]. Results are expressed as mean ± SEM. Comparison between the distribution of alleles was performed using a χ^2-test. A $p < 0.05$ was considered statistically significant. The study was approved by the board of ethics of the University of Duisburg-Essen and all patients and their parents gave their written consent.

3. RESULTS

A total of 126 patients were diagnosed as WCHT but diagnosis was confirmed by ABPM in only 48 patients. HT and WCHT patients were not different regarding height, weight, and BMI. Systolic and diastolic BP was significantly increased in hypertensive patients (systolic daytime141 ± 10 mmHg, diastolic daytime 83 ± 11 mmHg; systolic nighttime 127 ± 8, diastolic nighttime 67 ± 13 mmHg) when compared to 78 WCHT patients (systolic daytime 115 ± 14 mmHg, diastolic daytime 71 ± 10 mmHg; systolic nighttime 105 ± 14, diastolic nighttime 60 ± 8 mmHg).

A total of 126 patients were genotyped for the presence of the *ClC-Kb* T481S polymorphism (**Table 1**). Allelic frequencies for the A- and T-allele in hypertensive and WCHT patients were not significantly different between both groups (A-allele: HT 0.84 vs. WCHT 0.85; T-allele: HT 0.16 vs. WCHT 0.15) and did not differ from data obtained from population studies in the literature [8] (**Table 1**). The distribution of all genotypes was in Hardy-Weinberg equilibrium. When looking more detailed in the group of WCHT patients whose BP exceeds the 90th percentile, we identified 34 out of 78 patients (44%) fulfilling criteria of a borderline HT. In this subgroup, the allelic frequency was significantly shifted towards the T-allele (A-allele 0.71 vs. T-allele 0.29; $p < 0.05$ when compared to 0.15 in all other patients of this group), suggesting that patients at risk for the development of HT express the T-allele more often (**Table 1**).

Table 1. Allele and genotype frequency distribution of ClC-Kb T481S.

Genotype	HT (n = 48)	WCHT (n = 78)
T481T (homozygous noncarriers)	6 (75%)	58 (74.4%)
T481S (heterozygote carriers)	9 (18.8%)	17 (21.8%)
S481S (homozygous carriers)	3 (6.2%)	3 (3.8%)
Allele		
A-allele	(n = 81) 0.84	(n = 133) 0.85
T-allele	(n = 15) 0.16	(n = 23) 0.15
Allelic frequency of ClC-Kb T481S in WCHTchildren (RR > 90th percentile, n = 34)		
A-allele	(n = 48) 0.71	
T-allele	(n = 20) 0.29* vs. 0.15	

*$p < 0.05$, Fisher exact test and χ^2-test

4. DISCUSSION

Hypertension is a major risk factor for the development of cardiovascular and cerebrovascular complications such as heart attack and stroke [9]. Hypertensive end-organ damage has also been demonstrated in children and there is a huge scientific and economic interest in identifying children and adolescents at risk for developing HT in order to initiate preventive treatment [10]. Therefore, even if HT does not appear to be significant initially, children with borderline HT should have continuing follow-up of their BP and monitoring for the complications of HT. Our preliminary data suggest that children with borderline HT carry the ClC-Kb T481S polymorphism more often. The T481S polymorphism has been described to be the first mutation that leads to a pronounced gain of channel function and might therefore contribute to elevated BP [7,8].

We are aware of the limitations of our retrospective study such as small patients number and single center design. However, this is the first study to support the in vitro findings by Jeck and co-workers [7,8] that the

ClC-Kb T481S polymorphism detects children at risk for development of HT while few other studies in adult hypertensive patients did not identify a positive correlation so far [11]. Extended analysis and a larger population of children with marginal elevated BP values are required to clearly answer this question.

It is well known that renal tubular Na^+ and Cl^- transport mediate BP control by influencing the extracellular fluid volume [12]. Therefore, genes involved in the regulation of renal salt absorption are of potential interest as candidate genes for the development of renal HT. Jeck *et al.* [7,8] demonstrated that the *ClC-Kb* gene T481S polymorphism leads to activation of the chloride channel in the kidney. It is therefore feasible to hypothesize that also minor changes in the handling of renal salt absorption might lead to elevated BP. Proof of concept using chloride absorption would be interesting to study. However, due to the retrospective design of this study and the lack of established standards in renal tubular chloride handling in children, we are unable to test this hypothesis in our study population.

It is important to know that all patients investigated are European Caucasians which makes the possibility unlikely that a subpopulation with a different ethnical background exists Therefore, we can speculate that the increased prevalence of the T-allele in patients with borderline HT is feasible and not biased by their genetic background. However, other co-factors predisposing for HT such as obesity or metabolic syndrome cannot be ruled out [13]. In this respect it is interesting to notice that both, hypertensive and WCHT patients had an increased BMI exceeding 23 kg/m^2 making obesity a likely risk factor for development of HT in our study population.

In conclusion, children and adolescents with borderline HT carry the *ClC-Kb* T481S polymorphism more often and this genetic variant might predispose for the development of arterial HT. Larger scale studies in genetically diverse populations excluding other predisposeing factors of HT are needed to prove these observations.

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Vol.2, No.7, 685-691 (2010)
doi:10.4236/health.2010.27104

Using concept maps in cognitive treatment for children with developmental coordination disorder

Tsameret Ricon

Occupational Therapy Department. Faculty of Health & Welfare Sciences, University of Haifa, Mount Carmel, Israel; tricon@univ.haifa.ac.il

Received 13 January 2010; revised 20 January 2010; accepted 21 January 2010.

ABSTRACT

Children with Developmental Coordination Disorder (DCD) often seem to possess a narrow repertoire of cognitive strategies. In particular, they have difficulties in learning and internalizing the rules and strategies that other people intuitively use to approach common everyday problems. As a result, they often appear to have organizational, planning, memory and learning difficulties. The article proposes using a Concept Map (CM) as a visual strategy to facilitate interaction between a child with DCD, his/her family and therapist, as reflected in Client Centred and cognitive approaches. The CM is used as a method of assisting the child to identify, develop and utilize cognitive strategies in order to manage daily tasks effectively, as a tool in organizing his own therapy and in order to encourage participation. A demonstration of the concept mapping usefulness is brought by a case report. Further uses of concept mapping as a useful strategy within the framework of intervention remain to be studied.

Keywords: Concept Map; Cognitive Approach; Developmental Coordination Disorder; Organization Problems; Case Report

1. INTRODUCTION

Children with Developmental Coordination Disorder (DCD) experience problems with planning, ordering and then carrying out coordinated movements and tasks appropriate for their age. The use of cognitively-based interventions designed to help children with DCD to develop problem-solving strategies shows greater transfer to other areas of skill development than do traditional physical and occupational therapies [1]. This article aims to demonstrate the use of the concept map (CM) as a cognitive strategy for treating children with DCD.

A concept map consists of a central word or concept surrounded by a few main ideas that relate to that word. It is a graphical two-dimensional display of concepts, connected by directed arcs encoding brief relationships (linking phrases) between pairs of concepts forming propositions.

Concept maps serves as tools for organizing and representing knowledge. They offer a method to represent information visually and therefore harness the power of our vision to understand complex information "at-a-glance". We can assist the sequencing by which tasks are learned through attainment of progressively more explicit knowledge that can be anchored into developing conceptual frameworks.

Concept maps are, representation of concepts and their interrelationship, and are intended to represent the knowledge structures that humans store in their mind [2]. Concept maps are important when adopting a constructive view of learning. This view locates cognition and understanding within the individual [3], and is based on the theory that each of us develops mental schema that serve to inform future thinking or action [4]. It is thought that these schemas enable us to function with confidence in a complex environment, and it is these schemas that CM aims to represent diagrammatically.

In 1976, Novak [5] defined concept maps as a visual, organized representation of knowledge enhancing meaningful learning. Concept maps give structure to "the to-be-learned domain", including the before, during, and the after phases of action, thus playing an important role as an integrative organizer.

The use of cognitive-based approaches to enhance occupational performance (in the case of children with DCD, this is defined as their ability to successfully undertake their daily school, play, leisure and self care activities) has been a developing focus in contemporary occupational therapy literature [6]. In the early 1990s, Polatajko and colleagues [7] set out to develop a new approach to the treatment of children with DCD. Given the fact that children with DCD have difficulties in learning and generalizing motor skills, it seemed that the motor skills needed to be learned in more efficient way.

Meichenbaum [8,9] proposed that children could learn to regulate their behaviour by instructing themselves to identify a goal, develop a plan, enact the plan, and evaluate its success. In the Cognitive Orientation to daily Occupational Performance approach [10]; cognitive strategies are used to influence skill acquisition by children with occupational performance deficits. Generalization and transfer of skills is supported through the use of an executive or problem-solving strategy that trains the child to monitor his or her performance and self-evaluate the outcome. Domain-specific strategies form the bridge between the child's ability and skill level and help the child to develop appropriate motor plans.

1.1. CM as a DCD Intervention Tool

The behaviour of children with DCD often seems disorganized, presumably deriving from altered use of cognition, caused by otherwise biology (**Figure 1**) [11].

However we currently lack tools to directly assist children develop strategies that will enable them to become better organized at the cognitive level. In addition, to generalize and then transfer a learned strategy, the child must have knowledge of how, when and where to use that strategy [12]. Pressley and colleagues [12] recommend guided discovery learning as the optimal method for achieving transfer. CM, being a visual means of organizing cognition, can act precisely as such a learning method, and so assist children organize both their cognition and their behaviour, generalizing and transferring the learned strategies to other areas of their lives.

Intervention approaches have been mainly criticized for achieving only limited improvements for children with DCD in terms of generalized motor performance. An evaluation of the various techniques was performed by Mandich *et al.* [13], who found no difference, in terms of effectiveness, between Sensory Integrative (SI) therapy, physical education classes and perceptual motor treatment. A Meta analysis performed by Hen, Mayseles and Josman [14] analysed the mean effect size of the effectiveness of intervention approaches that had been reported in studies between 1987 and 2007, and found no significant differences in the effectiveness of the various approaches to treating children with DCD.

If we relate to DCD using Morton's model [11]—we see a biological defect that causes a cognitive consequence that manifests as a behavioural impairment (see **Figure 1**). Similarly, DCD interventions should aim at the biological, consequence, or impairment levels (**Figure 2**).

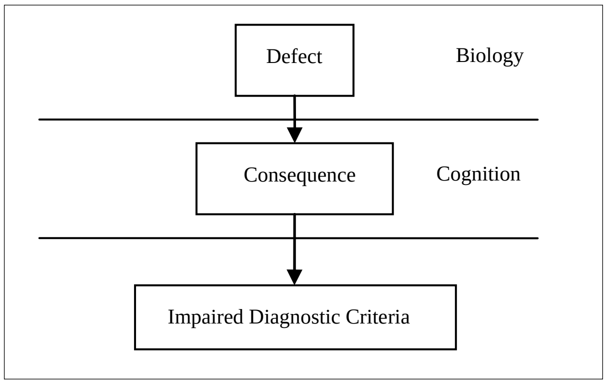

Figure 1. Morton's (2006) basic causal model.

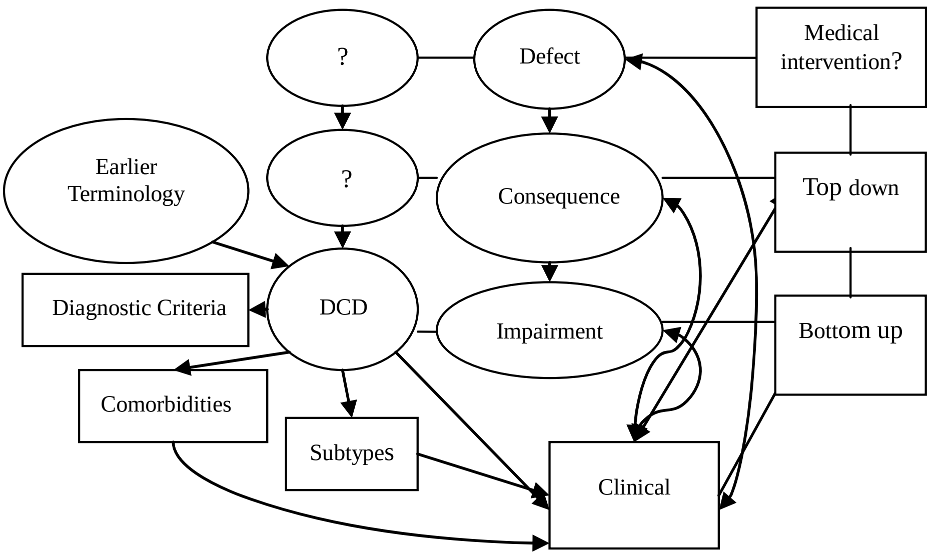

Figure 2. Concept Map of the Issues DCD Therapists must consider in making clinical Decisions.

Neuromaturational, hierarchical theories consequently led to focus therapy on remediation underlying deficits with the expectation of subsequent improvement in motor performance. Other approaches define the objectives of intervention as facilitating skill acquisition, and therapy focuses on functional splinter skills. However, they have not been found to be effective in improving the functional ability of children with DCD [1]. These approaches need to be integrated into interventions aimed at higher levels.

Top down approaches focus on the role that the context of motor behaviour plays in organizing the motor system for performance. Contemporary theories propose that behaviour is self-organized and emerges from various subsystems, and emphasize a problem solving approach to motor skill acquisition. Top down approaches included task specific interventions, and cognitive treatments [1].

Cognitive theory literature has shown the use of strategies to facilitate performance. According to the Morton [11] model (**Figure 1**), the cognitive level is above the behavioural level of carrying out a task Cognitive strategies are in the conscious cognitive level and are controllable.

Feuerstein, Hoffman & Miller [15] believed that children's cognition could be modified. Their cognitive deficiencies are observed either when the child approaches the task, thinks about the task or responds to it.

Consequently, Polatajko *et al.* [7] proposed a problem solving approach, which uses cognitive skills through verbal self guidance to improve the child's motor performance. In this top-down approach, the goal is the activity defined by the clients and objectives are: skill acquisition, transfer of skills across environments and tasks, and development of appropriate cognitive strategies [7]. **Figure 3** illustrates where concept mapping fits within interventions aimed at the consequence (cognitive) level and within the broader DCD context.

This original pilot study suggests a step-by-step, new problem solving approach—using concept mapping. The visual presentation of organized linked ideas about any activity that is about to be done plays a role in facilitateing cognitive strategizing. This approach can be used with any age and with problems of any complexity by everyone: the child/parent/therapist/teacher/case manager and so forth. An example usage of CM to aid a child in achieving a desired skill is shown in **Figures 4** and **5**.

Research into visual processing in children with DCD leads us to another controversial domain. Children with DCD experience difficulties in performance of all kinds

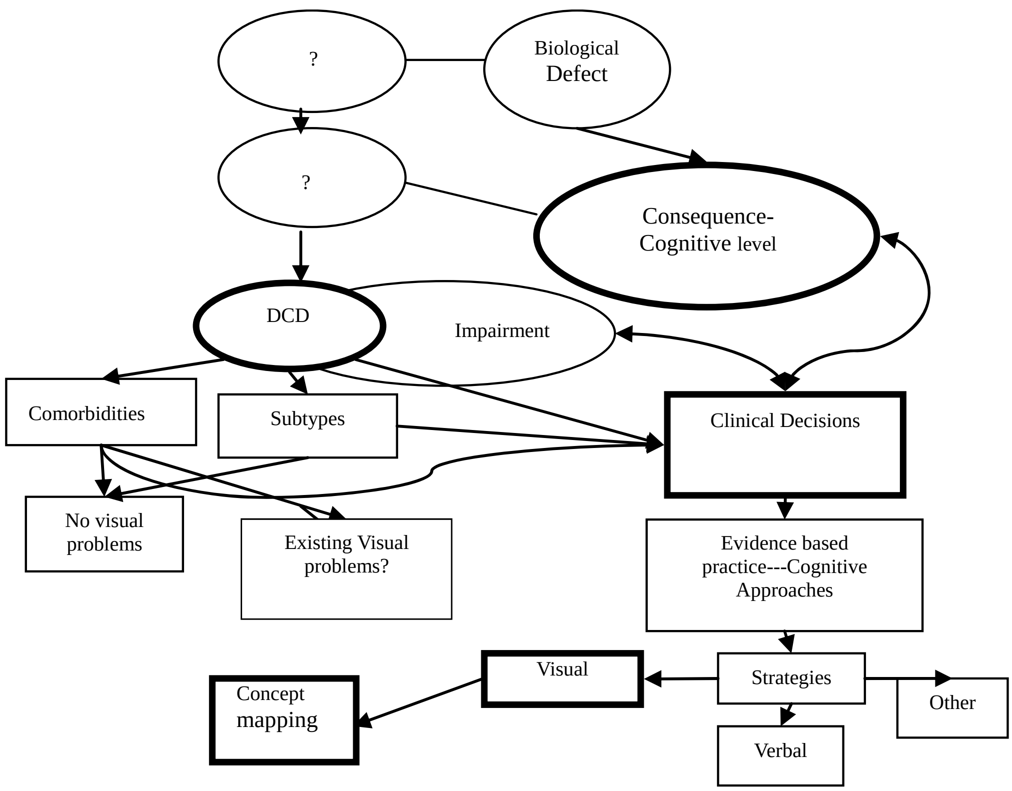

Figure 3. Clinical decisions deriving from the consequence level lead us to the use of cognitive approaches and concept mapping.

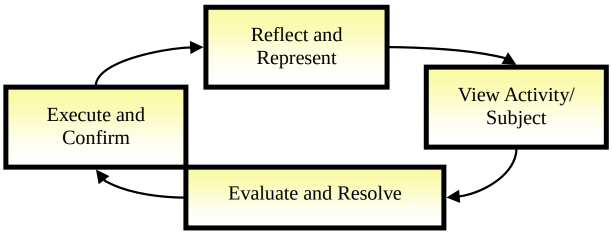

Figure 4. Phases of the constructed adoption of the visual strategy.

of motor skills, deficits in perceptual processes have often been assumed to underline these difficulties. Schoemaker and her colleagues [16] showed that children with DCD were not a homogenous group on the matter. They were as able as typical children to detect and match figures in a complex, confused background, and their most pronounced problems had motor nature. At this point the issue of co morbidity arises. Some studies that did show visual-perceptual problems failed to control for comorbidy and could not exclude the possibility of influence from other factors [17]. Another brief concept summary is presented in **Figure 3**.

Considering the overall picture with respect to DCD, and in light of the effectiveness of cognitive approaches, we suggest using the Concept Map as a cognitive visual tool in DCD intervention.

In the process of Concept Mapping, in parallel to the processes outline thus far, knowledge is anchored in conceptual frameworks that enable clients to develop more effective solutions to problems in occupational performance. Concept mapping can be used as a planning tool prior to the activity, as an organizational strategy during the activity, and as a reflective activity that answers the needs of both the therapist and the child.

Using Concept Maps requires explication (making explicit what is normally implicit). The child becomes more aware of the required regulation of his/her learning processes in relation to task performance and the abilities to be acquired. Both explication and awareness contributes to the development of auto-monitoring techniques/strategies [4]. The constructed adoption of the visual strategy is demonstrated in **Figure 4**.

In the first node, the child is asked to "View the activity" in a preliminary stage that includes a graphic presentation of information. The activity could be any activity or even any subject, such as planning study for an exam or planning how to climb a ladder. In both cases, the aim is to arrive at implement able actions, bringing together all knowledge and previous experience. The second node is "Evaluate and resolve", and relates to the actual planning part. This is the process of building up a concept map in order to serve thinking/action and to enable functioning in a complex environment. The child is asked to elaborate on the problem verbally. S/he must write down or tell the therapist all the required steps of the activity or sub topics of the main subject, which require attention. Furthermore, the child must internalize and understand the relation between the sub-topics and the dynamic structure of the map. The therapist can serve as a mediator during this phase, to the extent required by children at different levels.

The next phase is "Execute and confirm", meaning

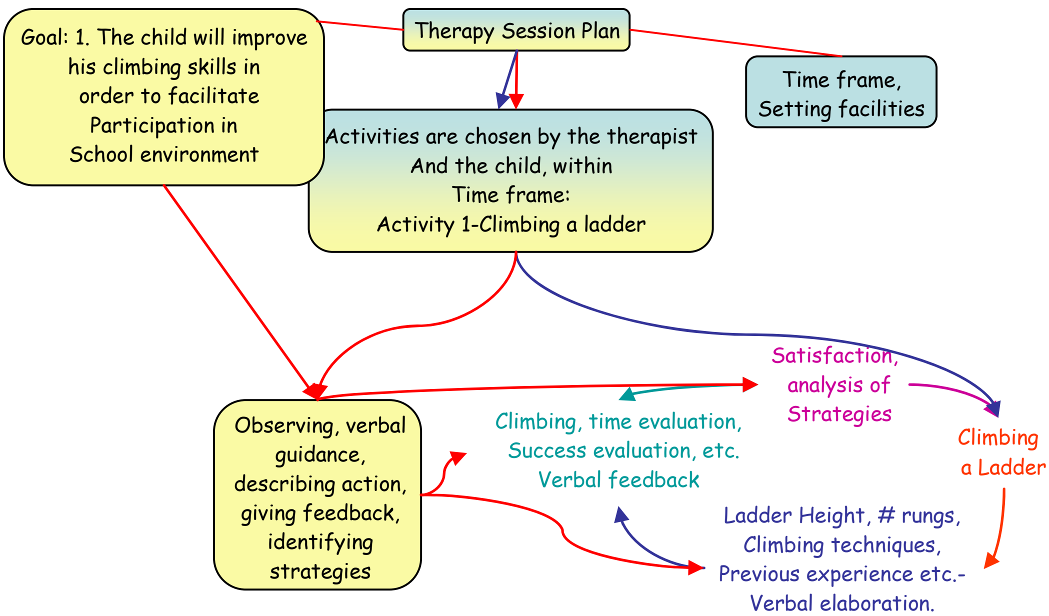

Figure 5. An example of concept map used in therapy.

that the child executes as planned, and the therapist supports the child's actions with verbal guidance and reinforcement. During the last phase, "Reflect and represent", both the child and the therapist function as a "cognitive mirror". The child must reflect on his/her actions, organization and structure. With the assistance of the therapist, the child tries to manage cognitive resources more effectively and to establish/refine new/existing strategies.

An example of practical management of a CM is shown in **Figure 5**. The concept map must be a context related ("what are we going to do today") map. It has to have familiar content. We must ensure that the child recognizes the relationships between concepts (show him/her an example of a relationship, and let him point out a new one). And it is created by the child and therapist together, that is, it is "client cantered". After decideing with the child upon the activities to engage, the child elaborates on the activity verbally, make plans, draw them and so forth. Then execute and reflect on his performance.

The therapist has indirect control over the client's motivation in choosing to learn by attempting to incorporate new meanings into their prior knowledge, rather than simply memorizing the concept or plan. CM can function as an easy-to-use tool that enables the therapist to assess how the child is learning, where and how cognitive errors are occurring, and to undertake an overall evaluation of the learning strategies being taught.

2. CASE STUDY

2.1. "Yoav"-An 8 Year Old Boy with DCD

"Yoav" (the original name is kept confidential) an 8 year old boy was diagnosed by a physician as having DCD. He was referred to occupational therapy in order to relate to his organizational problems manifested in school and at home environments. Yoav has normal intelligence (IQ $\leq$ 85), no deficits in hearing and vision, and is right-handed. He and his parents had signed a parental consent and agreement to participate in therapy sessions using "concept map strategy", after they where explained about it. They also agreed to the presentation of this case report, anonymously.

Yoav did not get previous or other present cognitive-based treatment for his motor problems. Yoav was not diagnosed with any neurological disorder, physical or other sensory deficits.

3. METHOD

The tests that were administered during clinical intake included the Movement Assessment Battery for Children Test (M-ABC); [18] Movement Assessment Battery for Children (Manual). Sidcup, Kent, UK: The Psychological Corporation. Yoav scored less than the 15th percentile, suggesting a DCD diagnosis.

3.1. Pre- and Post-Measures Used to Evaluate Treatment

In order to evaluate Changes in the tasks performance, identified by Yoav's perspective of himself, we used the Canadian Occupational Performance Measure (COPM) [19]. Canadian occupational performance measure. Toronto, Ont.: CAOT Publications A. The COPM is a semistructured interview designed to help clients identify problems in occupational performance. Yoav was asked to identify two major tasks out of five to be worked on during therapy. Using a 10-point scale, Yoav rated the tasks on perceived performance and satisfaction. The COPM was repeated after treatment to evaluate perceived change in performance and satisfaction.

Baseline data for the two tasks that Yoav defined were obtained prior to treatment. Performance baseline for each task was obtained for three repetitions of each of the two tasks. Yoav had chosen: 1. organizing his school bag 2. Making plans for a complex activity (such as studying for an exam, planning a trip). His performance seemed unorganized, unplanned and unsatisfying for him in terms of "end product" and self image.

3.2. Treatment

Over 10 individualized sessions, Yoav learned the concept mapping strategy and then applied it in performing better organization of trip planning and his school bag. Using the Concept mapping strategy, Yoav had learned to disassemble the task to its components and reorganize the task sequence and process through problem solving.

3.3. Treatment Protocol

The protocol of learning and implementing the concept map strategy in a chosen activity-planning a trip will be presented here : the overall sessions were built according to the phases of acquiring the visual strategy-"View the activity", "Evaluate and resolve", "Execute and confirm", "Reflect and represent".

Two first sessions were dedicated to learning the idea of concept-mapping strategy. On this stage we exercise different kinds of concept maps (family tree, human senses etc.) and made sure that Yoav could add new concepts or nodes and understood their relations, for example-we drew the basic family tree and ask him to add an extra horizontal node (in the same horizontal level of hierarchylike a brother or sister) and a vertical node (which is a different level in hierarchy like great grandmother above grandmother).

Session 3-5: gathering information for the trip plan to the desert. Organizing information becomes more important as the amount of information increases. Yoav and the therapist wrote down every trip planning component (equipment, maps, dates, tracks, food requirements, entertainment activities…) and made a graphic representation of them using computer flow charts (**Figure 6**).

Each time, adjustments were made. A new node or new connectors were added to the map. Parts that needed more information such as "tracks" were marked down and led to a new information quest. The quest usually yielded new nodes and new connectors, and usually new problems to solve (delays in driving hours, shortage of food and water supplies...).

Session 6 and 7: Defining responsibility domains according to the map: After finalizing map demands and domains, Yoav had to elaborate on it to his family members and give each member a responsibility domain. This presentation process strengthened his awareness and shown his broadening knowledge. Members of the family raised adequate questions.

This stage demonstrated the learning process-gathering knowledge-remembering, recognizing and identifying. Comprehension-interpreting, translating from one medium to another, describing in one's own words, organization and problem solving-selection of facts and ideas, use of facts, analysis of rules and principles and Synthesis *i.e.* creating an original product (the concept map), forming a new ensemble using ideas that come from the analysis process.

Sessions 8-10: executing the plan and evaluating its success. On this phase, Yoav was requested to make value decisions about his plan; develop an opinion and judge decisions, and resolved ambiguity regarding the trip plan.

3.4. Post Test

After the completion of treatment, The COPM was re-administered to Yoav. All activities were videotaped for future analysis.

4. RESULTS

Treatment effects were tested by comparing pre-test and post-test scores. The COPM performance and satisfaction ratings of Yoav were averaged across Yoav two goals, yielding an average performance rating and an average satisfaction rating. For both performance and satisfaction, improvements from pre-test to post-test were achieved (**Table 1**).

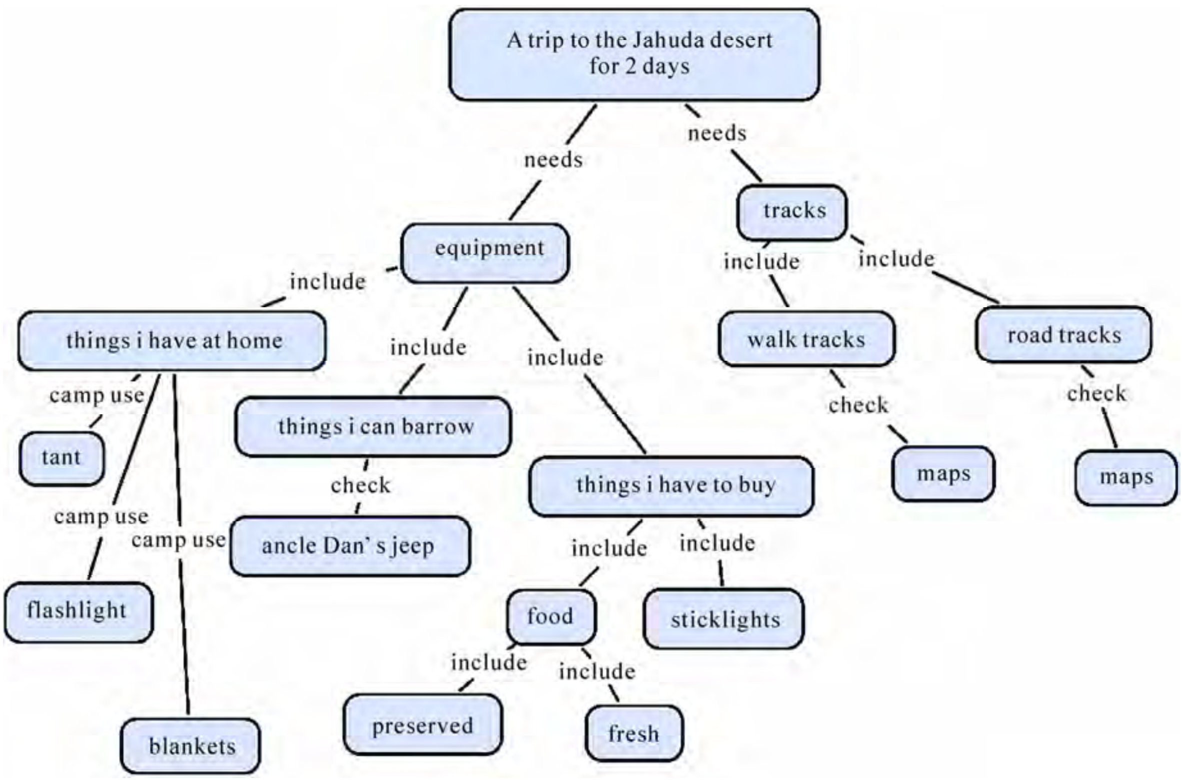

Figure 6. One of Yoav's concept maps in preparation and planning of the trip (translated from Hebrew).

Table 1. COPM satisfaction and performance mean scores pre and post intervention.

Score	Pre-test M	Post test M
COPM performance	4	8
COPM satisfaction	3	9

5. CONCLUSIONS

The purpose of this article was to demonstrate the use of the concept map as a visual cognitive strategy in the treatment of children with DCD, and it's utility on as shown on a case report.

Similarly to other cognitive approaches, the use of concept mapping focuses directly on child-performance issues, and engages the child as an active problem solver and a significant partner in the therapy process. The results although limited to the case study and were done in a limited pre-post analysis, are encouraging with regard to the effectiveness of the concept mapping, and indicate that further uses of concept mapping as a useful visual strategy within the framework of intervention for children with DCD remain to be studied.

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Vol.2, No.7, 692-695 (2010)
doi:10.4236/health.2010.27105

Health

Are mean platelet volume and splenomegaly subclinical inflammatory marker in children with familial mediterranean fever?

Ismail Dursun[1]*, Faysal Gok[1], Oguzhan Babacan[2], Erkan Sarı[2], Onur Sakallıoglu[1], Suleyman Kalman[1], Ibrahim Gokce[2]

[1]Department of Pediatric Nephrology and Rheumatology, Gülhane Military Medical Academy, School of Medicine, Ankara, Turkey;
[*]Corresponding Author: drdursun@hotmail.com
[2]Department of Pediatrics, Gülhane Military Medical Academy, School of Medicine, Ankara, Turkey; faysalgok@yahoo.com, gokcemd@yahoo.com

Received 6 February 2010; revised 2 Mrach 2010; accepted 5 March 2010.

ABSTRACT

The present study aimed to investigate the correlation of MPV and splenomegaly as inflammation activity of FMF patients at the attacks free period. We retrospectively reviewed the medical records of 43 patients with FMF. This study was performed at the attack free period as clinical and laboratory. For this study, patients were divided into two groups. Patients with splenomegaly is called group 1 (n = 12) and patients with no splenomegaly is called group 2 (n = 31). Groups were compared respect to age, gender, platelet counts, acute phase reactants and MPV. The mean MPV (fl) were significantly higher in group 1 (8.9 ± 0.8) than in group 2 (8.4 ± 0.5, p < 0.05). This study suggested that increased MPV and splenomegaly without amyloidosis could be a sign of chronic inflammation in children with FMF even in attack free period.

Keywords: Familial Mediterranean Fever; Mean Platelet Volume; Subclinical Inflammation

1. INTRODUCTION

Familial Mediterranean fever (FMF) is a recessive disorder characterized by attacks of periodic fever and inflammation [1]. The FMF patients are asymptomatic, acute phase reactants (C-reactive protein, ESR) are normal in the attacs free period, but serum amyloid A (SAA) and some cytokines are constantly higher [2,3]. Subclinical inflammation continues in attack-free period in FMF patients [3,4].

It has been reported that IL-6 /HPRT is significantly higher than control in attack free period of FMF (3). IL-6 has been shown to promote megakaryocyte maturation in the absence of other growth factors. It possibly stimulates megakaryocyte proliferation. Administration of IL-6 has been shown to result in a significant increase in the platelet count [5]. Also it is a cytokine that can induce the formation of SAA and C-reactive protein (CRP) [6-8]. Erythrocyte sedimentation rate, CRP and SAA correlate closely with clinical disease activity in FMF patients [4].

The spleen acts as a reservoir for platelets; one-third of the circulating platelet mass is temporarily sequestered within a normal sized spleen, and up to 90 percent may be found within a markedly enlarged spleen. The etiology of splenomegaly may relate to an increase in a normal splenic process (e.g. hemolysis) or may be due to infiltrative, infectious, or vascular disorders [9]. Involvement of the reticuloendothelial system in FMF is rare mentioned in the literature [10,11]. Splenomegaly in patients with FMF may be related to the rate of platelet destruction. The changes of peripheral platelet volume depend on the rate of splenic sequestration and bone marrow production. The platelet survival is directly related to the lowest platelet count and inversely related to both the highest mean platelet volume and duration of the thrombocytopenia [12]. If the rate of platelet destruction is equal or higher than the rate of platelet production, mean platelet volume is increase because of increased peripheral immature platelets. If the rate of platelet destruction is lover than the rate of platelet production, mean platelet volume is normal or decrease [12,13].

Mean platelet volume (MPV) is a parameter generated by full blood count analyzers as part of the routine complete blood count (CBC) test which is usually overlooked by clinicians. MPV correlates with the platelet function and activation. An association between FMF activity and MPV has not been investigated yet.

The present study aimed to investigate the correlation of MPV and splenomegaly as the inflammatory activity of FMF patients at the attacks free period.

2. PATIENTS AND METHODS

We retrospectively reviewed the medical records of 43 patients with FMF who were followed up in our Pediatric Nephrology and Rheumatology Unite. The diagnosis of FMF was made according to the diagnostic criteria which described by Livneh *et al*. *and* DNA analyses [14,15]. This study was performed at the attack free period. The attack free period was accepted if the clinically patient had no symptoms and in the laboratory, ESR and CRP were normal. Patients were divided into two groups according to splenomegaly. Patients with splenomegaly is called group 1 (n = 12) and patients with no splenomegaly is called group 2 (n = 31). Groups were compared respect to age, gender, platelet counts, acute phase reactants (ESR and CRP) and MPV. None of the patients had abnormal urinalysis for proteinuria. Splenomegaly has been established by physical examination and confirmed by abdominal ultrasound (USG). In USG examination, we used age and height dependant standarts for splenomegaly [16].

3. STATISTICAL ANALYSIS

All tests were performed using SPSS for Windows 15.0. The parameters with normal distribution were expressed as mean ± SD and the parameters with abnormal distribution were expressed as median (minimum-maximum). Comparisons of means were performed with unpaired t-test. Comparisons of medians were performed with Mann-Whitney U-test. Comparisons of proportions were performed with Pearson-chi-squared test. A P value < 0.05 was accepted as statistically significant.

4. RESULTS

The results of genotype of the patients with FMF were showed in **Table1**. **Table 2** shows epidemiological and laboratory findings of the patients with FMF. While the group 1 included two girls and ten boys, the group 2 included 15 girls and 16 boys. The mean age of the group 1 and group 2 were 11.0 ± 4.4 and 10.1 ± 3.5, respectively. The mean platelet counts of the group 1 and group 2 were 248500 ± 67654 and 281000 ± 51847, respectively. There was no significant difference between group 1 and group 2 according to age and platelet counts (p > 0.05). The mean MPV (fl) were significantly higher in group 1 (8.9 ± 0.8) than in group 2 (8.4 ± 0.5, p < 0.05) (**Table 2**).

Table 1. Shows genotyping distribution of patients with FMF.

MEF Mutation	Group1 (n = 12)	Group 2 (n = 31)
M694V/M694V (n)	5	8
M694V/N (n)	3	8
M694V/V726A (n)	1	2
M694V/M680I (n)	2	1
V726A/R761H (n)	1	-
E148Q/N (n)	-	3
V726A/V726A (n)	-	1
M680I/V726A(n)	-	1
M694V/E148Q(n)	-	2
V726A/N(n)	-	2
Normal (n)	-	3

Table 2. Epidemiological and laboratory findings of the patients with FMF.

Variables	Patients with splenomegaly (n = 12)	Patients with no splenomegaly (n = 31)	p
Age (years)*	11.0 ± 4.4	10.1 ± 3.5	> 0.05
Gender (M/F)	10/2	16/15	0.05
Platelets counts (× 10^3/mm^3)*	248 ± 68	281 ± 59	> 0.05
Mean platelet volume (fl)*	8.9 ± 0.8	8.4 ± 0.5	< 0.03

Mean ± SD

5. DISCUSSION

Involvement of the reticuloendothelial system in FMF is seldom mentioned in the literature [10,11]. Enlargement of the spleen without amyloid deposition has been reported in 30.7% of patients with FMF [17]. Aharoni *et al.* [18] detected splenomegaly in 27.5%, 13.3% of patients with FMF at the attack and at attack free period, respectively. In our study, splenomegaly was detected by physical examination and abdominal ultrasound in 27.9% of FMF patients at the attacks free period.

In literature, so far, it has not been reported study that investigates association between MPV and disease activity in patients with FMF. There are few studies about the association between MPV and the clinical activity of rheumatoid arthritis. Milovanovic *et al.* [19] have reported that in active disease IL-6 but not thrombopoietin (TPO) is related to platelet count. Thus, IL-6 raises platelet count in reactive thrombocytosis and the neutrophil count. In rheumatoid arthritis, MPV and myeloperoxidase also mirror the disease activity [19]. In the other study has been reported that cytokines and IL-6, IL-11 and growth factors (e.g. TPO) may also contribute to the pathologic megakaryocytopoiesis of RA [20]. Kisacik *et al.* [21] have been shown the correlation between MPV and the clinical activity of rheumatoid arthritis and ankylosing spondylitis. They suggest the assessment of MPV that may provide additional information about inflammation in AS and RA.

Up now, there are two study that has been shown the association with splenomegaly and genotype in patients with FMF in the literature [22,23]. Kone *et al.* [22] showed that homozygosity for the M694V mutation correlated with splenomegaly. But, Inal *et al.* [23] didn't detect the association with splenomegaly and genotype in patients with FMF. In our study, since the the number of the patients is small to do the correlation with the genotype and splenomegaly, we do not find the result statistically significant.

In conclusion, the diagnosis of FMF is based mainly on the clinical criteria and laboratory examinations. We found the correlation of MPV and splenomegaly as the inflammatory activity of FMF patients at the attacks free period. These could help to diagnosis of the FMF and may be applible for clinical chronic inflammatory condition score marker that related to prognosis or the possibility that development of amiloidosis. Further and including much more number patients studies are needed to confirm.

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Vol.2, No.7, 696-704 (2010)
doi:10.4236/health.2010.27106

Health

Disabling hip osteoarthritis: gender, body mass, health and functional status correlates

Ray Marks

School of Health & Behavioral Sciences, City University of New York, York College, and Department of Health & Behavior Studies, Columbia University, Teachers College, New York, USA; rm226@columbia.edu

Received 12 February 2010; revised 15 March 2010; accepted 16 March 2010.

ABSTRACT

Objective: To examine gender differences in self-reported pain and function before and after hip replacement surgery and the extent to which overweight, comorbidities and muscular status impact pain and function in adults with disabling end-stage hip joint osteoarthritis. Setting: Orthopedic Hospital Setting on the East Coast of the United States. Study Design: Cross-sectional retrospective chart review. Methods: The desired demographic, physical and psychological attributes of 1040 adults with end-stage hip osteoarthritis hospitalized for hip surgery were recorded and subjected to comparison and correlational analyses. These data included gender, self-reported weight, height, numbers and nature of physical and psychological comorbidities, pain intensity, ambulatory capacity and discharge destination. Sub-group analyses of 808 candidates hospitalized for primary unilateral surgery were also conducted using SPSS 16. Results: There were significant ($p < 0.05$) associations between gender, pain scores, comorbidity numbers and ambulatory capacity. Specifically, women who exhibited higher comorbid disease rates than men, exhibited higher pre-surgery pain levels and greater functional limitations in walking ability before and after surgery than men with the same condition. In sub-group analyses of men and women with the same mean age, comorbid prevalence rates, and body mass indices, women were found to have significantly higher ideal weights on average than men, and those with higher ideal weights recovered more slowly after surgery ($p < 0.05$). Conclusion: The presentation of hip joint osteoarthritis is not uniform, and may be impacted differentially by gender. Women with high ideal body weights, may be specifically impacted. Whether genetic or other factors account for gender differences in pain and function among adults with disabling hip osteoarthritis observation needs to be examined.

Keywords: Body Mass; Comorbidity; Function; Gender; Hip Joint; Osteoarthritis

1. INTRODUCTION

Hip joint osteoarthritis, a prevalent medical condition, causes considerable distress and chronic disability among community-dwelling adults aged 55 years and older in all countries. Associated with substantial direct as well as indirect costs, a number of factors other than aging may influence its impact. These factors include the presence of one or more comorbid health conditions [1] varying degrees of pain [2], variations in the disease itself [3], gender [4], high body weights [5], as well as proprioceptive, muscle strength and hip flexion range of motion deficits. In addition, prior musculoskeletal injuries, excessive occupational stresses and systemic factors may impact the severity of osteoarthritis [5].

Because hip osteoarthritis, a leading cause of functional disability [4] is related to poor-self related health [6], and causes great suffering, but is often approached quite uniformally as far as treatment is concerned [7], it was felt a better understanding of the disease and its clinical heterogeneity [8] might prove beneficial. In particular, it was felt that if further evidence for important subsets in the expression of the disease could be identified, more targeted efforts to reduce the significant social and economic burden of the disease might be forthcoming in the future. Factors such as prevailing mental and physical impairments, other than hip osteoarthritis and their interaction with pain and functional capacity have also received limited attention in the related literature [3].

To bridge this gap, this work examined the association between gender, pain and function and potentially preventable or treatable comorbid health conditions, obesity, and lower leg weakness as experienced by a hospitalized cohort of community dwelling adults with end-stage hip osteoarthritis. More specifically, this analysis attempted to discern if there are any clinically relevant gender differences in the pain experience and functional status of individuals of similar sociodemographic backgrounds and disease severity, and whether this was impacted by the presence of prevailing age, body mass, comorbid health conditions, and other factors such as muscle strength capacity.

The primary research questions driving this investigation were the following:

1) Is the extent of the disability experienced by adults with disabling hip joint osteoarthritis similar for men and women?

2) What coexisting health conditions are most commonly observed among community dwelling adults with end stage hip joint osteoarthritis and is this uniform for men and women?

3) Is the level of pain and mobility experienced by adults with disabling hip joint osteoarthritis influenced by body mass and/or the presence of one or more pre-existing medical conditions?

4) Is the level of pain and reduced mobility experienced by adults with disabling hip joint osteoarthritis influenced by the presence of muscle weakness as estimated on a numeric scale?

2. MATERIALS AND METHODS

All available medical records of community-dwelling adults hospitalized for hip replacement due to clinically and radiographically diagnosed end-stage hip osteoarthritis of one or both hips were examined. These data had been collected prospectively over a 10 month period as part of an approved parent study of hip joint surgical outcomes, but with no active patient involvement. Patients with acute hip fractures or any other primary diagnosis were excluded.

2.1. Study Sample

The cohort examined included 1040 men and women between the ages of 23-89 years diagnosed as having definitive clinical and radiographic evidence of osteoarthritis of one or both hip joints requiring primary or secondary surgery as determined by an orthopedic surgeon. The majority came from the local community.

2.2. Procedures

The desired data were systematically extracted by the researcher from the patient's medical record. These data, which had been systematically recorded by the attending physician[s], nurse[s] and physical therapist, included age, gender, self-reported height and weight. They also included the primary reason for hospital admission, the presence and nature of any pre-existing orthopedic problems, comorbid physical and/or mental health conditions other than hip disease, and symptomatic self-reported baseline pain estimates on a 5-point ordinal scale where 1 was minimal pain and 5 was maximal pain. Muscle weakness of the affected limb as identified by a composite of physical tests including manual muscle tests of the knee and hip muscles rated from 5-1 (no problem-severe loss of strength) and active hip flexion joint range of motion estimates of the affected hip (in degrees), plus patient perceived pre-operative walking status and distance in blocks and 3-day post-operative walking distance as recorded in meters by the physical therapist were also recorded. Separate calculations on the chart included those of body mass index (weight/height^2) and ideal weight estimates (calculated for each individual based on gender, age, height, and weight from standard estimates that represent average medically recommended values) and expressed as a percentage of the patient's actual body weight. Also recorded was the disease duration, as well as presence of any other affected joints and prior surgeries. The data were entered systematically onto an Excel spreadsheet and transposed thereafter, into SPSS version 16.0 files to describe the sample and to analyze the data with regard to age (above and below 60 years of age), gender, comorbid health numbers, and pain and functional variables among the observed hip osteoarthritis surgical candidates using chi-square tests, cross-tabulations, and analysis of variance, as indicated. An a priori significance level of 0.05 was adopted.

3. RESULTS

3.1. General features

The 1040 cases presently studied had a mean age of 65.36 ± 13.04 years, with a median age of 68 years, and a mode of 71 years. As shown in **Table 1**, approximately 30% or 312 were 60 years or under in age. The number hospitalized for primary unilateral surgery was 808 or approximately 78%, 53 or 5% required bilateral surgery, and 179 or 17.2% were undergoing revision surgery. Overall, the present sample was constituted by more females than males (60% vs 40%), but among cases under the age of 60, the numbers of hospitalized men and women were comparable (158 vs 163). Among this cohort, there were 9 reported comorbid health domains related to different body systems as outlined by Dyke *et* *al.* [1], excluding obesity, including those shown in **Table 2**. The frequency of patients having reported either a mental or physical pre-existing health condition ranged from 0 or no co-existing problem to 5, with a mean of 1 ± 1, and a median of 1; 36% had no comorbid conditions, 30.5% had 1 condition, 22.9% had 2 conditions, 6.7% had 3 conditions, 1.2% had 4 health conditions, and 0.2% had 5 co-existing conditions. The majority or 69.5% of cases had at least one additional pre-existing physical health condition and the numbers of reported comorbid health conditions increased with age ($r = 0.232$; $p = 0.001$). That is, those who were 60 years and older had more evidence of the presence of one or more chronic health conditions than those below 60 years of age.

When analyzed separately with regard to gender, on average, women tended to slightly more comorbid conditions than men (CIs being 1.06-1.22; 0.83-1.00, respectively), although the median of 1, and range 0-5 were comparable ($X^2 = 18.61$; $df = 15$; $p = 0.232$). In addition to the high prevalence of having at least one co-existing physical problem, 52.7% of the cohort studied had a pre-existing orthopaedic problem such as a fracture, or congenital bone problem, and in contrast to medical comorbidity prevalence, men had higher rates of co-existing orthopaedic related problems than women ($X^2 = 9.98$; $df = 9$; $p = 0.001$). Women had higher rates of autoimmune diagnoses related to arthritis ($X^2 = 9.42$; $df = 9$; $p = 0.001$), and cardiac disease including hypertension, heart murmurs and defective valve conditions were observed most commonly among the diseases listed on the charts.

In comparing those requiring bilateral hip surgery with those hospitalized for primary unilateral surgeries, these patients were found to be younger on average with a mean age of 54.11 ± 13.18 years versus a mean age of 66.03 ± 12.4 years. Their comorbidity level was also

Table 1. Summary of key baseline demographic and disease related characteristics of the hip osteoarthritis cohort. Values are means and standard deviations and percentages of total sample.

Characteristics	Overall Group N = 1037	Women N = 600	Men N = 437
Demographics			
Age (years)	65.36 ± 13	67.22 ± 12.96	62.74 ± 12.70*
# under 60 yrs	316 (30.4%)	153	163
# over 60 yrs	723	447	274*
Body mass index ($kg.m^{-2}$)	27.5 ± 5.7	26.6 ± 5.9	28.7 ± 5.1*
Morbidity Count	1.0 ± 1.0	1.2 ± 0.04	0.92 ± 0.04*
(Range 0-5)			
0	37%	19%	17.5%
1	31.2%	18.9%	12.3%
2	23.4%	13.5%	9.9%
3	6.8%	5.1%	1.7%
4	1.3%	1.2%	0.2%
5	0.2%	0.1%	0.2%
Concomitant orthopedic problem	52%	25.2%	34%*
Autoimmune related diagnosis	5.6%	4.5%	2.2%*
Depression history	6.4%	5%	1.4%*

*men were significantly younger than women admitted for the same surgery p = 0.001
*fewer men over 60 yrs were admitted than women in present cohort p = 0.001
*men had fewer reported comorbid conditions (p = 0.002)
*men had higher body mass indices in general than the women, as well as more orthopedic problems than women p = 0.001
*men had fewer autoimmune and depression history related diagnoses than women p = 0.001

Table 2. Summary of extent of comorbid diseases noted on charts at baseline among hip osteoarthritis surgical cases when disaggregated by gender showing significant gender differences (N = 560).

	GENDER	
Comorbid condition	**Female** N (%)	**Male** N (%)
Cardiovascular disease	187 (33%)	124 (22%)
Hypothyroidism	47 (8.3%)	5 (0.76%)
Osteoporosis	46 (8.8%)	1 (1.8)
Cancer	36 (6.4%)	22 (3.9%)
Depression	28 (5%)	8 (1.3%)
Autoimmune disease	28.5 (5%)	13 (2.3%)
Diabetes	20 (3.5%)	13 (2.3%)
Asthma	16 (3.9%)	20 (2.8%)
Stroke	5 (0.75%)	0
Kidney disease	4 (0.71%)	0
Prior injury/bone defect	98 (17.5%)	91 (16.2%)

Note: Some cases have more than one comorbid disease.
No records of visual or hearing impairments were available.
Cardiovascular disease category includes high blood pressure, or any pre-existing heart condition or vascular disease.

lower than those undergoing unilateral surgery (p = 0.001), and was independent of age, when assessed using cross tabulations. It was thus deemed of interest to specifically examine only those undergoing unilateral surgery, as it was felt, those with bilateral hip joint osteoarthritis may have had a different health profile, in general from the majority of the primary surgical cases. Those hospitalized for a previous procedure, revision or re-operation were also excluded.

3.2 Related Subgroup Analyses

In a further analysis of the 808 cases undergoing primary unilateral surgery, median age, 68 years, it was observed that this sub-group had one comorbid health problem on average and the majority of these cases or 69.2% were overweight or obese with a mean body mass index of 27.6 ± 5.5. Among those unilateral cases with comorbid health conditions, the most common condition observed was hypertension alone, or in combination with coronary artery or cardiac disease. Next, injuries, bone disease or prior surgeries were reported most often. The other conditions were hypothyroidism, osteoporosis, and cancer. Women had higher rates of all conditions except asthma and stroke, and lower rates of prior injury than men.

To better control for potential confounders, a further sub-analysis of 100 age-matched cases, mean age 66.3 ± 10.3 years (66.52 ± 10.98 years of age for the 46 women; 66.06 ± 9.69 years of age for the 54 men, p = 0.822), with comparable body mass indices of 27.7 and 27.9, for women and men, respectively (p = 0.432), and an average of 1.2 comorbid conditions was undertaken as shown in **Table 3**. Again, this analysis revealed the subjective pain levels experienced before surgery were found to be significantly higher among the women compared to the men (3.2 ± 0.84 versus 2.7 ± 0.9 on a 5 point Likert scale, respectively) (p = 0.004), even though numbers of affected joints were comparable (p = 0.125). As well, men in the cohort tended to be able to walk further on average than women before surgery, as well as three days after surgery (p = 0.011; p = 0.018, respectively). While these differences could not be explained on the basis of comorbity numbers or body mass index ($w.h^{-2}$) because comorbidity rates were comparable, and a similar percentage were overweight or obese (48% men vs 50% women), the ideal body weights estimates calculated for women were 134 percent (CI 113-155) and 106 percent (CI 89-123) for men (p = 0.002) with a mean of 124.8 + 25.3 for the group. There was also a significant inverse relationship between the ideal weight estimates and the ability to walk one day after surgery (r = –0.246, p = 0.043), but post surgical walking distance was not affected by age, pain, comorbid status or body mass. On the whole, weakness of the lower leg was evident in 40% cases, and of these cases, 7% had weakness of both the hip and knee muscles. Although walking endurance at baseline was generally worse if the knee extensors and or the hip and knee extensors on the operative side were found to be weak, this was not significant (p = 0.196). More subjects however, used devices to help them walk if they reported weakness of the lower leg (p = 0.009), and a higher percentage of women exhibited a lower leg strength loss than men (61% vs 34%). Although the cohort were clearly challenged physically speaking, and disease histories extended from 1 year to 18 years, pain levels were not influenced by disease duration and only 5% women and 1.3% men had depression histories. Walking distance and stair walking was affected by numbers of affected joints, but was independent of muscle strength. Device use was correlated positively with age.

4. DISCUSSION

Hip joint osteoarthritis, a debilitating condition that increases in prevalence with age, presents an enormous challenge to the health care system worldwide due to its chronicity. To improve the outcomes for this group of

Table 3. Table showing relationships between gender and walking capacity before and after surgery when controlling for pain in a sub-group of cases older than 60 years of age (N = 100).

Gender	Age Yr	Walk Dist Blocks	Comorbid Number	Day 3 walk dist Feet	BMI w.h^{-2}	Ideal wt Percent
F N = 46	66.5 ± 10.9	2.71 ± 2.89	1.11 ± 0.89	4.95 ± 43.1	5 27.2 ± 5.2	132.8 ± 25.3
M N = 54	66.1 ± 9.7	4.61 ± 3.9**	1.24 ± 0.8	88.78 ± 43*	27.9 ± 4.2	118.6 ± 18.7**

** correlation is significant at 0.01 level (2-tailed)

* correlation is significant at the 0.05 level (2-tailed)

adults, careful evaluation of the underlying pathophysiological and contributing factors to the disease has become essential. To this end, this present retrospective analysis examined trends and interrelationships that might exist among selected physical and health related correlates of the condition and patient characteristics believed to impact on osteoarthritis disability. In particular, a better understanding of mediating, moderating, or causative factors such as gender, body mass, comorbid health status, and muscle weakness and the clinical parameters of pain and functional disability was sought. The goal was to identify new perspectives or to further support current perspectives about what may be most appropriate for purposes of reducing the disability and handicap of hip joint osteoarthritis.

To this end, patients with symptomatic end-stage hip osteoarthritis, and well defined radiological progression were selected for study as there has been no universal agreement as to what stage of progression might be highly important to examine in the context of the natural history of the disease, and which has been poorly evaluated in the past [8]. All had well defined clinical as well as functional disability that had progressed towards its end stage, and the present study examined the variation in disease presentation as regards health status, gender, functional ability and pain among other factors to improve our understanding of this condition. Patient subsets were identified and examined to ascertain if any would be more affected clinically by the disease than others, and if so, if these displayed any unique physical and health status characteristics.

While the current retrospective chart review approach can be seen as limitation, this exploratory study tried to include the entire population of patients with active disease who were admitted for primary or secondary hip surgery over a 10 month period and examined their short-term functional recovery rates post-surgery. Although it is recognized that the present findings could be biased by who was studied, the age range of the studied cohort was quite typical of those described in the literature [7,9] and consistent with several other studies [7,10], a high percentage were women. As well, a high majority of the cases presently reviewed reported having at least one co-existing comorbid physical health condition, a finding identified by Van Dijk *et al.* [2] for adults with osteoarthritis of the hip and knee. Moreover, consistent with findings by Tuminen *et al.* [9], those older than 60 years of age had significantly higher comorbidity rates than those under 60 years of age (See **Table 4**).

In addition, although contrary to findings of Franklin *et al.* [11] in an Icelandic case control study, a consistently high percentage of the study cohort were overweight or obese as was observed by Dijk *et al.* [2]. While obesity can be regarded as a comorbid health condition in its own right [2,8], this health indicator was presently analyzed separately from the other reported comorbid health conditions, along with estimates of the subject's ideal body weight estimates expressed as a percentage. In this respect, more than two thirds of the men and women presently studied were overweight or obese and women had higher percentages of ideal weight estimates than men when controlling for age, body mass, and comorbidity number, suggesting women with this disease are more overweight in general than men, when considering their height and age. In addition, there were gender differences in pre-surgical pain levels and functional mobility at baseline and post-surgery, in favour of the men, and different distributions of comorbid health conditions between men and women.

The higher than desirable numbers of comorbid health conditions, autoimmune conditions, and high body weights especially among women, are important to note, because, as has been observed in other studies, hip osteoarthritis is more frequently part of a polyarticular disease, with greater symptomatic and structural severity [7]. In addition, comorbidity and high body mass are risk factors for functional activity limitations and pain [2,3,9] and greater deterioration of hip joint disease [12]. Obesity is also correlated with higher mortality rates [11] and poorer health status. While the severity of the comorbidity was not studied, consistent with findings by Van Dijk *et al.* [2] the most common coexisting disease presently observed was heart disease or hypertension, a health condition expected to influence both activity and pain levels, as well as the outcomes of options for surgery and rehabilitation.

Table 4. Sample of past studies that support some of the present findings and the hypothetical disability model.

Authors	Sample	Key Findings
Jorring [13]	6321 cases of which 4.7% had hip OA	In all age groups above 60 hip OA was twice as common in women as in men
		The condition was more severe radiologically in women who more handicapped than men
Juahkoski *et al*. [14]	118 cases of hip OA	Comorbidity (CM) number influenced pain/function
Juhakoski *et al*. [15]	840 cases hip OA	Heavy manual labor + injury were predictive for hip OA
Kadam *et al*. [16]	11375 OA cases	CM for OA was extensive compared to controls
		Non musculoskeletal conditions observed included obesity, heart disease, phlebitis
		CM was not explained by age, gender or social class
Katz *et al*. [17]	Patients undergoing hip	Women had worse functional status than men surgery for hip OA
Maiilefert *et al*. [7]	508 patients with hip OA	Hip OA in women is more frequently part of a polyarticular OA and displays greater symptomatic and structural severity
Roseman *et al*. [18]	1250 OA patients	Main predictor of disability included body mass
		Impact of OA differed between genders
		More women than men constituted the sample
Tepper *et al*. [19]	73 cases of hip OA	Age/hip trauma were associated with hip OA in men Obesity was associated with bilateral hip OA
Tuominen *et al*. [9]	893 patients	649 or 73% had CM, mean no. = 2 -At baseline health-related QOL was lower in those with CM
Van Dijk *et al*. [2]	288 elderly-hip/knee OA	Almost all had at least comorbidity
		CM was related to activity limitations/pain
		Most common CM condition = cardiac diseases Overweight and obesity was common

Note: CM = comorbidity; OA = osteoarthritis; QOL = quality of life

In terms of gender, and as has been observed in other studies, although men and women had an equal chance of being included in the study, women clearly constituted the majority of those hospitalized over the study period. Moreover, even though the prevalence of the disease is probably similar among men and women [7], consistent with findings of other studies [7] women of comparable ages to men with similar health histories, could not walk as far on average with the same condition either before or after surgery. These observations support the view that being female may be an independent risk factor for disabling hip osteoarthritis as outlined by O'Connor [4] and the higher rates of endocrine/etabolic diseases, as well as cardiac diseases among the women may explain their performance-based activity limitations [1]. They may also suffer from higher rates of muscle mass and strength losses due to one or more of these health conditions, as well as high levels of fat infiltration that might impact their ability to function physically as they age [20]. Indeed, although the women and men currently examined had comparable body mass indices, women as a whole exhibited higher ideal body weight estimates as well as higher rates of strength losses of the affected lower leg that can limit performance [1]. They also had higher rates of reported depression and pain.

Men, on the other hand, were more likely to simply be overweight, to have lower rates of comorbid disease and-to have suffered a prior orthopaedic problem, suggesting a somewhat different disease profile. They may thus suffer less or have less widespread intercurrent diseases and related impairments than women and are thus more active functionally before surgery and able to recover more rapidly after surgery, and in the present case were generally younger than the women.

Alternately, the greater disability presently experienced by women than men with the same diagnosis, which has also been observed among knee arthroplasty candidates [21], may indicate women who are candidates for hip joint replacement surgery should be preferentially targeted before and after surgery to offset unwarranted functional declines, and the possibility of developing further radiological damage, cardiac diseases and others. The data also show considerable inter-individual differences in disease presentation and suggest more attention to classifying patients according to the presence or absence of comorbidities and mechanical or biological factors may influence the disease outcome as well as body weight, pain and functional capacity quite favorably [14,20-24].

Moreover, because untreated or poorly treated hip osteoarthritis may increase the risk for obesity, as well as related medical conditions, more carefully designed preventive and therapeutic strategies that focus on muscle strengthening are clearly indicated. In addition, to reduce the rate of progression of the disease, and to help the patient to function optimally, those with unrelenting pain might be targeted for treatment early on [6,25]. The higher rate of thyroid disorders, osteoporosis, and autoimmune conditions experienced by the women in the present analysis suggests additional targets for early intervention as well to offset possible excessive tissue damage and injury. As well, generic efforts to prevent injuries such as falls, may be influential in minimizing the hip osteoarthritis burden, as may more targeted efforts to reduce occupational stresses at work thought to lead to hip osteoarthritis [5,15].

However, it is recognized that the present cohort may not represent all cases with condition; not all data were available or complete; and chart reviews may not be without limitations. Also, even though several comorbid health problems and their frequency of occurrence were recorded, their severity was not. Visual impairments as well as hearing impairments were also not recorded for the hip osteoarthritis cases were studied. These conditions, and their prevalence and relationship to hip osteoarthritis disability thus need to be studied further to assess if concerted efforts to prevent and treat comorbid conditions, as well as depression and pain may lessen the disability associated with hip joint osteoarthritis and its negative impact on functional independence and life quality.

In summary, factors placing adults at risk for hip osteoarthritis disability are age, being female [10], having high body weights [11], and one or more comorbid health conditions. Muscle strength loss, trauma, excessive loading of the hip joint as well as congenital hip problems may also place individuals at risk for hip osteoarthritis as well high rates of further disability. Because over a third of the present cases were under 60 years of age, and had not necessarily experienced trauma, but were heavier on average than those over age 60 years, early and carefully tailored interventions to maximize weight control seems highly desirable. Moreover, given that being obese increases the risk for hip osteoarthritis 2.47 fold among women [26], women might be preferentially targeted. In addition, professionals interested in improving the outlook for adults with hip joint osteoarthritis must acknowledge the broad spectrum of other health problems adults with hip osteoarthritis may face that can impact the need for hip joint replacement [23] as well as postoperative recovery processes [12,27] and the true burden of the disease [28]. In light of its im-

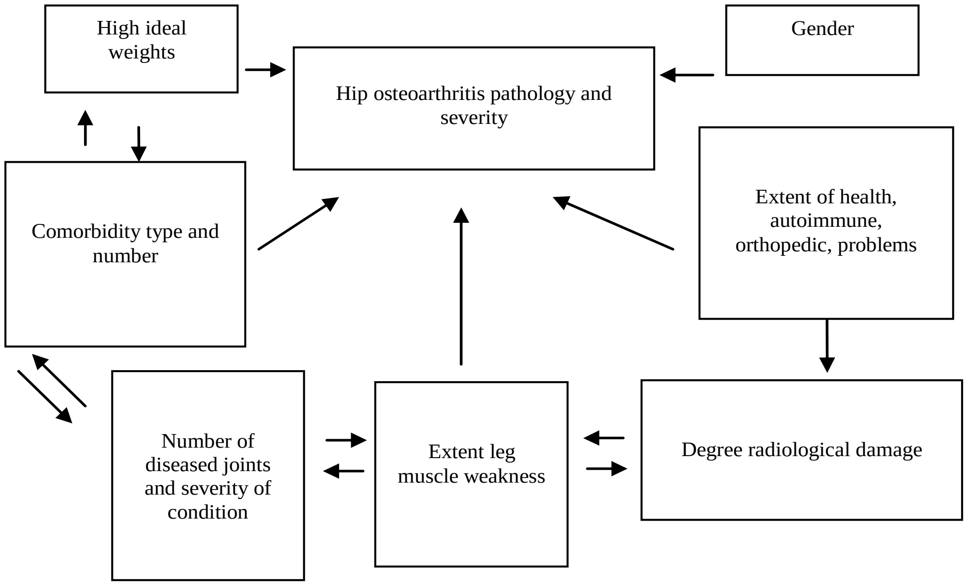

Figure 1. Hypothetical interaction of key variables other than age that might influence outcome of hip osteoarthritis.

mense variability, and disproportionate impact on women, a comprehensive rather than a uniform clinical pathway for intervening in the hip osteoarthritis degenerative process as advocated by Wang *et al.* [24] is indicated. In particular, in addition to targeted efforts to assist with weight maintenance or reduction and pain and depresssion control, the efficacy of implant and rehabilitation tailoring should be explored. Assessing comorbidities and the extent and severity of these at baseline may also help to prioritize medical-decision making [9,26] and to thereby tailor care approaches that can potentially reduce hospital stays, as well as the human and economic impact [13,16-19,25,29].

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Vol.2, No.7, 705-707 (2010)
doi:10.4236/health.2010.27107 **Health**

Pharmacotherapeutic aspects of treating knee osteoarthritis with glucosamine sulfate

Steven Simoens*, Gert Laekeman

Research Centre for Pharmaceutical Care and Pharmaco-economics, Katholieke Universiteit Leuven, Onderwijs en Navorsing, Leuven, Belgium; *Corresponding Author: steven.simoens@pharm.kuleuven.be

Received 5 February 2010; revised 11 March 2010; accepted 15 March 2010.

ABSTRACT

Glucosamine sulfate is a natural constituent of cartilage and is used in the treatment of knee osteoarthritis. The aim of this study is to provide a short but comprehensive pharmacotherapeutic update on treating knee osteoarthritis with glucosamine sulfate. A literature search was conducted of PubMed, Centre for Reviews and Dissemination databases, Cochrane Reviews and EconLit up to January 2010. The literature review indicated that the mechanism of action of glucosamine sulfate is based on hypothesis, but its treatment effects in knee osteoarthritis are symptomatic. With steady-state peak concentrations at the 1,500 mg dosage in the range of 10 μM, it is estimated that only 2% of glucosamine is incorporated in the cartilage. A once-daily dosage of 1,500 mg of glucosamine sulfate is licensed for the treatment of symptomatic osteoarthritis and has been shown to reduce pain, improve function and exhibit similar safety to placebo. Glucosamine sulfate is likely to be a cost-effective treatment of knee osteoarthritis. In conclusion, a once-daily dosage of 1,500 mg of glucosamine sulfate is likely to be a safe, effective and cost-effective treatment of knee osteoarthritis as compared to placebo.

Keywords: Pharmacotherapy; Glucosamine Sulfate, Knee Osteoarthritis; Pharmacokinetics; Indication; Safety; Effectiveness; Cost-Effectiveness

1. INTRODUCTION

Knee osteoarthritis is associated with significant morbidity, impaired quality of life, and substantial healthcare costs [1]. Pharmacological treatment of knee osteoarthritis includes analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and opiods, and glucosamine sulfate. Glucosamine sulfate is a natural substance that is a constituent of cartilage. Glucosamine sulfate is sold as a dietary supplement in the United States, but is registered as a medicine throughout much of Europe.

To date, there is uncertainty over various pharmacotherapeutic aspects of treating knee osteoarthritis with glucosamine sulfate. Therefore, the aim of this study is to provide a short but comprehensive pharmacotherapeutic update on treating knee osteoarthritis with glucosamine sulfate.

2. METHOD

2.1. Search Strategy

A literature search was conducted of PubMed, Centre for Reviews and Dissemination databases, Cochrane Database of Systematic Reviews and EconLit up to January 2010. Additionally, the bibliography of included studies was checked for other relevant studies. Search terms included "knee osteoarthritis", "knee joint", "glucosamine sulfate", "pharmacokinetics", "bioavailability", "indications", "dosage", "safety", "effectiveness", "cost-effectiveness", "economic evaluation" alone and in combination with each other.

The literature search included articles published in peer-reviewed journals and congress abstracts. Also, the researchers contacted manufacturers of glucosamine sulfate for any unpublished studies. The review was limited to studies published in English for practical reasons.

2.2. Inclusion and Exclusion Criteria

The review was limited to the use of glucosamine sulfate in its indication of knee osteoarthritis.

To explore the pharmacotherapy of glucosamine sulfate from a multidisciplinary perspective, the literature review assessed the following aspects: pharmacokinetics, indications and dosage, safety and effectiveness, and cost-effectiveness. Evidence about cost-effectiveness was derived from economic evaluations. An economic evaluation was defined as a study comparing glucosamine sulfate with an alternative treatment in terms of both costs and consequences.

3. RESULTS

3.1. Search Results

The results of the literature search are displayed in **Figure 1**. The review summarized three studies on pharmacokinetics, a Cochrane literature review on safety and effectiveness, and three economic evaluations of glucosamine sulfate in knee osteoarthritis.

3.2. Pharmacokinetics

A literature review analysed the absorption, distribution, metabolism and excretion of different administration forms of glucosamine sulfate [2]. The absolute bioavailability of oral glucosamine sulfate amounted to 44%. The fecal excretion was 11.3% of the dosage, indicating that at least 88.7% of the dosage was absorbed through the gastrointestinal tract. With an absolute bioavailability of 44% calculated after oral 14C-glucosamine, a first-pass effect can be hypothesized.

A study examined the dosage-proportionality of glucosamine sulfate in 12 healthy volunteers [3]. The pharmacokinetics were linear in the 750 mg and 1,500 mg dosages, but not at 3,000 mg, where the plasma concentration-time profiles were less than expected based on dosage-proportionality. More recent research showed that glucosamine concentrations in plasma and synovial fluid were correlated [4]. There remains controversy over whether steady-state peak concentrations at the 1,500 mg dosage were in line with those found to be effective in selected in vitro studies.

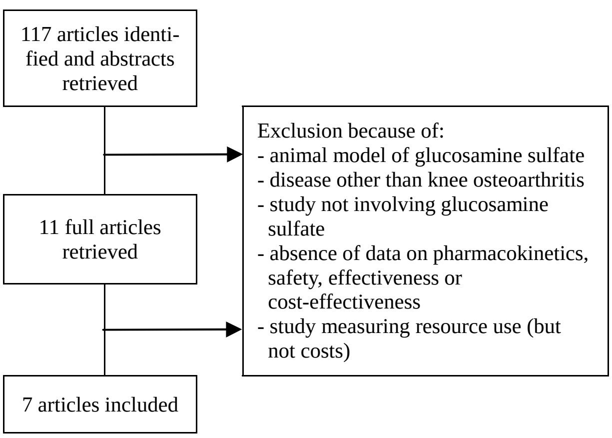

Figure 1. Flow chart of literature search.

3.3. Indications and Dosage

The indication of glucosamine sulfate in Europe is limited to the symptomatic treatment of knee osteoarthritis. An expansion of indication to osteoarthritis of the hip, spine, hands and other locations was refused, as a result of which some companies have refused to register their preparation as a medicine. Glucosamine sulfate can be used alone or in combination with NSAIDs. The licensed dosage is 1,500 mg, once daily. Symptom relief may not be experienced until after several weeks of treatment, and optimal effects on joint mobility have been observed after 12 weeks of administration.

3.4. Safety and Effectiveness

A Cochrane review assessed randomized controlled trials (RCTs) evaluating the effectiveness and toxicity of glucosamine in osteoarthritis up to the end of 2008 [1]. Of the 25 studies included in the review, 20 RCTs focused on the knee exclusively and five RCTs examined osteoarthritis at other or multiple sites. All studies were double-blinded randomized parallel-group trials, enrolling a total of 4,963 patients (mean age of 60.7 years, 69% were female). Fourteen RCTs had affiliations with Rotta Pharmaceuticals (an Italian manufacturer of glucosamine sulfate).

A pooled analysis of the relevant RCTs showed that, as compared to baseline, glucosamine sulfate taken orally in amounts of 1,500 mg/day produced an increase in the total score of the Lequesne Index (a multidimensional index measuring knee pain or discomfort, activities of daily living and maximum distance walked) of 11% (standardized mean difference of –0.47; 95% confidence interval: –0.82 to –0.12). A subgroup analysis of the Rotta preparation showed significant benefit over placebo in terms of pain (standardized mean difference of –1.11; 95% confidence interval: –1.66 to –0.57) and in terms of the total score of the Lequesne Index (standardized mean difference of –0.47; 95% confidence interval: –0.82 to –0.12). Studies using a non-Rotta preparation failed to show benefit over placebo in terms of pain and function as measured by the Western Ontario and McMaster Universities Index (a disease-specific index measuring pain, stiffness and function). Two RCTs suggested that the Rotta preparation of glucosamine sulfate may slow radiological progression of knee osteoarthritis over a three-year period (average difference of 0.32; 95% confidence interval: 0.05 to 0.58).

Glucosamine therapy exhibited a safety profile similar to placebo in terms of the number of patients with side-effects (relative risk ratio of 0.99; 95% confidence interval: 0.91 to 1.07) and significantly better than NSAIDs (relative risk ratio of 0.29; 95% confidence interval: 0.19 to 0.44).

3.5. Cost-Effectiveness

A Belgian economic evaluation indicated that treatment of knee osteoarthritis with glucosamine sulfate for at least one year and up to three years was associated with a lower incidence of total knee replacement over an observation period of eight years [5]. Costs of glucosamine sulfate treatment were offset by lower hospitalization costs, resulting in overall savings. Total costs per patient amounted to € 1,103 in the placebo group and € 901 in the glucosamine sulfate group. In other words, treatment of knee osteoarthritis with glucosamine sulfate for up to three years is associated with savings of € 202 per patient during the overall observation period of eight years (i.e. up to three years of treatment and five years of follow-up). This study indicated that glucosamine sulfate treatment was more effective and less expensive than placebo in knee osteoarthritis. If costs of other osteoarthritis medicines and other health care resource use are taken into account, the cost-effectiveness of glucosamine sulfate would even be more favourable.

The National Institute for Health and Clinical Excellence in England and Wales compared the likely cost-effectiveness of selected treatments for knee osteoarthritis [6]. This study did not provide a full economic evaluation, but presented UK healthcare costs alongside the effectiveness of each treatment option. The scope of costs was limited to treatment costs (such as medicine costs), but did not include other healthcare costs such as adverse event costs or decreased use of other medical resources. The results suggested that glucosamine sulfate (1,500 mg/day) is likely to be cost-effective as compared with placebo.

A recent US analysis explored the cost-effectiveness of glucosamine sulfate treatment versus placebo in a hypothetical cohort of 100,000 patients who were 65 years old and had knee osteoarthritis for five years [7]. A hybrid Markov-decision tree model was constructed using published effectiveness data and US fee schedules. Treatment with a daily dosage of 1,500 mg of glucosamine sulfate turned out to be more effective and less expensive than placebo by delaying time to joint replacement surgery. However, cost-effectiveness results were sensitive to changes in several input parameters and the authors recommended additional research to inform decision making.

4. CONCLUSIONS

This study provided a pharmacotherapeutic update on treating knee osteoarthritis with glucosamine sulfate. Additionally, an economic evaluation explored the cost-effectiveness of treating knee osteoarthritis with glucosamine sulfate in Belgium. The main messages can be summarized as follows. The mechanism of action of glucosamine sulfate is based on hypothesis, but its treatment effects in knee osteoarthritis are symptomatic. A once-daily dosage of 1,500 mg of glucosamine sulfate has been licensed for the symptomatic treatment of knee osteoarthritis. Glucosamine sulfate has been shown to reduce pain and improve function of patients with knee osteoarthritis. Glucosamine sulfate therapy exhibits a safety profile similar to placebo and significantly better than NSAIDs. Finally, glucosamine sulfate is likely to be a cost-effective treatment of knee osteoarthritis.

5. ACKNOWLEDGEMENTS

No funding was provided to support this study. The authors have no conflicts of interest that are relevant to the content of this manuscript.

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Vol.2, No.7, 708-712 (2010)
doi:10.4236/health.2010.27108

Health

Platelet aggregation responses in type 2 diabetic patients

Fatma Mutlu Kukul Güven[1]*, Abdülkerim Yilmaz[1], Hüseyin Aydin[2], Ilhan Korkmaz[1], Sevki Hakan Eren[1]

[1]Emergency Medicine Deparment, School of Medicine, Cumhuriyet University, Sivas, Turkey;
[*]Corresponding Author: fmkg@hotmail.com
[2]Biochemistry Lab, School of Medicine, Cumhuriyet University, Sivas, Turkey; haydin@cumhuriyet.edu.tr

Received 12 February 2010; revised 12 March 2010; accepted 14 March 2010.

ABSTRACT

Diabetes mellitus (DM) is associated with platelet dysfunction. In diabetic patients, alterations in platelet functions, especially increased platelet agregation, have been suggested to cause increasing in cardiovascular morbidity and mortality or in accelaretion of athersclerotic process. In this study, we aimed to investigate the platelet aggregation response alterations and the effects of DM duration, HbA_{1c}, treatment options among the patients with Type 2 DM. Forty-five patients (case group; 21 male, 24 female) with Type 2 DM and forty-eight healthy individuals (control group; 22 male, 26 female) were included in this study. Platelet aggregation was determinated with Chorono-log 500 (USA) named device by using Chorono-log/ADP, Chorono-log/collagen and Chorono-log/epinephrine kits. ADP-induced platelet aggregation was significantly higher in the case group compared with control group ($p < 0.05$). Epinephrine induced platelet aggregation were significant in negatively correlation with the diabetes duration ($P < 0.05$). Platelet aggregation responses did not differ according to their treatment type (sulphonylurea or insulin) was statistically insignificiant among the case groups ($p > 0.05$). In conclusion, our findings supported that type 2 diabetes may interfere with platelet functions without any relationship age, gender, the treatment types and the regulation levels. These findings supports that existence potential new factors or mechanism affecting platelet agregation. The subject requires more detailed studies in the future.

Keywords: Platelet Aggregation; Diabetes; Insulin; HbA1c

1. INTRODUCTION

Evidencies for abnormal platelet functions in diabetes mellitus (DM) have been shown as: altered platelet functions [1,2], increased aggregation of platelet that leads to acceleration of atherogenesis [1], abnormal platelet activation suggested to cause micro or macro angiopaties [2-4] and platelet hyperactivities [5,6].

The aim of this study was to investigate the platelet aggregation response alterations and the effects of DM duration, HbA_{1c}, treatment options among the patients with Type 2 DM.

2. METHOD

This study was performed in Cumhuriyet University Medicine Faculty Emergency Department between January-December 2003.

Study population: Forty-five patients (case group; 21 male, 24 female) with Type 2 DM diagnosis and forty-eight healthy individuals (control group; 22 male, 26 female) were included in this study. Case and controls had not any other systemic disease except type 2 DM. Neither of the patient of case group was diagnosed as type 1 DM. nor of the participitants had a treatment history by a drug that interferes platelet aggregation.

Blood sampling: A fasting state venous blood sample were taken in vaccuated tubes for all participitans and send to biochemistry laboratory within 30 minutes to measure blood glucose and HbA_{1c}. For platelet aggregation responses measurement venous blood samples were send to hematology laboratory within 30 minutes in 0.2 ml citrates containing tubes.

Study procedure: Blood glucose and HbA1c were studied by standart laboratory methods. Platelet aggregation was determinated with Chorono-log 500 (USA) named device by using Chorono-log/ADP, Chorono-log/ collegen and Chorono-log/epinephrine kits.

Statistical analysis: Data analysis were performed on SPSS (Ver 13.0) software by using chi-square, studen-t, Mann-Whitney U tests and correlation analysis.

3. RESULTS

The mean age was 58.68 ± 1.37 years in the case group and 53.72 ± 2.10 years in the control group. When case and control groups were statistically compared upon to their mean age and gender the difference was insignificant ($P > 0.05$) (**Table 1**). The mean glucose levels in the case and control groups were as 224.44 ± 15.95 mg/dl and 99.16 ± 11.84 mg/dl. The mean HbA1c level in the case group was 9.59 ± 0.38 mg/dl. The mean duration of diabetes in the case group was 8.06 ± 0.83 years. Sulfonylurea drugs were used in 44.45% [20] patient and insulin was preferred in 55.5% [25] patient for treatment.

Platelet aggregation responses induced with epinephrine, collagen and adenosine diphosphate (ADP) were measured and mean results were recorded as percentage(%) for both groups. The difference between the case and control groups were statistically insignificiant in terms of platelet aggregation responses induced with epinephrine and collagen ($p > 0.05$). Whereas; ADP-induced platelet aggregation was significantly higher in the case group compared with control group. (**Table 2**).

Platelet aggregation responses induced with all three activators were not in correlation with the ages in the both groups. (**Table 3**).

Platelet aggregation responses induced with all three activators were in negatively correlation with the diabetes duration. However, the correlation were significant for only epinephrine ($P < 0.05$). (**Table 4**).

When the platelet aggregation responses induced with three activators in the case groups were compared with HbA1c levels and the difference were not significant in statistical analysis ($p > 0.05$). (**Table 5**).

Platelet aggregation responses did not differ according to their treatment type (sulphonylurea or insulin) and statistically it was insignificiant among the case groups ($p > 0.05$). (**Table 6**).

4. DISCUSSION

Platelets functions are significant to understanding the pathophysiology of vascular disease in diabetes. The role of hyperglycemia is not clear in platelet hyperactivity in diabetic patients [7].

Platelet dysfunction may develop before vessel wall damage in diabetes [8,9]. Platelet dysfunction in diabetes, including altered adhesion and aggregation, is hypersensitivity to agonists [10].

Patient with type 2 DM had altered platelet functions and increased platelet aggregation responses with agonists [11,12].

Table 1. Epidemiological and laboratory properties of case and control groups.

	Cases $\bar{x} \pm S_e$	Controls $\bar{x} \pm S_e$	P
n (f/m)	45 (24/21)	48 (26/22)	$p > 0.05$
Mean Age (year)	58.68 ± 1.37	53.72 ± 2.10	$p > 0.05$
Mean time of DM (year)	8.06 ± 0.83	-	-
Treatment (OAD/ins)	20/25	-	-
HbA1c(mg/dL)	9.59 ± 0.38	-	-
Blood glucose (mg/dL)	224.44 ± 15.95	99.16 ± 11.84	$P < 0.05$

Table 2. Comparison of platelet aggregation responses in the case and control groups.

Groups	Epinephrine induced platelet aggregation (%)	Collagen induced platelet aggregation (%)	ADP induced platelet aggregation (%)
Case	45.75 ± 2.26	54.82 ± 2.76	74.91 ± 3.61
Control	42.43 ± 2.78	51.39 ± 2.00	55.72 ± 1.77
p	$t = 0.91$ $p > 0.05$	$t = 1.01$ $p > 0.05$	$t = 4.85$ $p < 0.05$

Table 3. Correlation between the age and platelet aggregation in the case and control groups.

Age	Epinephrine (%)	collagen (%)	ADP (%)
Case 58.68 ± 1.37	$r = 0.11$ $p > 0.05$	$r = 0.03$ $p > 0.05$	$r = 0.13$ $p > 0.05$
Control 53.72 ± 2.10	$r = -0.11$ $p > 0.05$	$r = -0.09$ $p > 0.05$	$r = -0.07$ $p > 0.05$

Table 4. Correlation between Diabetes duration and platelet aggregation in the case group.

	Epinephrine (%)	Collagen (%)	ADP (%)
Diabetes duration 8.06 ± 0.83	$r = -0.31$	$r = -0.23$	$r = 0.11$
	$p < 0.05$	$p > 0.05$	$p > 0.05$

Table 5. Correlation between HbA1c levels and the platelet aggregation in the case group.

Case Group	HbA_{1c}(mg/dl)				P
	5-7 n = 5	**7-9 n = 17**	**9-11 n = 10**	**11- ↑ n = 13**	
Epinephrine (%)	48.40 ± 9.30	46.29 ± 3.04	41.60 ± 5.28	44.23 ± 4.48	KW = 4.21 p > 0.05
Collagen (%)	60.80 ± 3.74	52.88 ± 3.98	54.90 ± 7.23	55.00 ± 5.96	KW = 2.71 p > 0.05
ADP (%)	72.80 ± 4.1	72.94 ± 6.27	68.10 ± 9.74	83.53 ± 5.82	KW = 4.21 p > 0.05

Table 6. Correlation between treatment type and platelet aggregation in the case group.

case	**Oral Antidiabetic Drugs n = 20**	**Insulin n = 25**	**p**
Epinephrine (%)	47.70 ± 3.23	44.20 ± 3.17	p = 0.367 p > 0.05
Collagen (%)	56.90 ± 4.35	53.16 ± 3.59	p = 0.775 p > 0.05
ADP (%)	76.75 ± 5.54	73.44 ± 4.84	p = 0.698 p > 0.05

In this study, three different activators caused to three different aggregation responses and significantly increased aggregation response was predicted for only ADP. This may depend on the presence of different possible diabetic complications. Because, the presence of diabetic complications may cause to variations in the platelet agregations response. However, the diabetic complications were not examined in this study and we failed, therefore, to conduct an exact conclusion on this subject.

The finding of increased aggregation response by ADP in diabetic patients is in aggrement with the finding reported previously [7,11,13,14]. Altough it was reported previously that there was a positive correlation between the age and platelet aggregation responses [15,16]. We failed to predicted such a correlation in this study. Knobler *et al*. [1] found no relationship between the platelet aggregation responces and the age of type 2 DM. Interestingly, we found a significant negative correlation between the platelet aggregation responses induced with epinephrine and diabetes duration. This finding suggest that there are possible new factors or mechanisms other than already known, having potence to interfere with platelet aggregation responses.

Mean platelet volume (MPV) is a marker of platelet function and activation. Larger platelets are more reactive and aggregable. Therefore it can be said that is a relationship between platelet function and diabetic complacations [17-20].

Increase in HbA1c concentration, indicative of worsening glycemic control, was accompanied by increased mean platelet volume which reflects deterioration of platelet function [21]. Whereas Hekimsoy *et al.* [22] did not found any correlation between HbA1c and MPV.

We found no relationship between HbA1c levels and the platelet aggregation responses; in aggrement with the findings of Mandal *et al*. [23] and of Hughes *et al.* [24]. Konya *et al.* [25] was found ADP-induced platelet aggregate were significantly reduced in the group with improved HbA1c.

The previous studies, except one [26], showed evidencies that oral antidiabetic drugs (OAD) have improving effects on platelet functions [27-30]. Another study was found that in the patients with improved glycemic control, gliclazide could inhibit ADP-induced platelet aggregation via the serotonin pathway [25]. Some studies suggests that insulin may inhibit platelet function at physiological concentrations [31,32], but enhance platelet aggregation at supraphysiological concentrations [33,34]. However; Hu *et al.* [35] had found that even physiological concentrations of insulin enhance platelet activation both in healthy subjects and in type I diabetic patient.

In our study, we found no significant difference in platelet aggregation responses regarding to the treatment type; insulin or OAD.

In conclusion, our findings supported that there several abmormalities in the platelet aggregation responses in type 2 DM patients and that the differences are not in correlation with control levels of blood glucose. The subject requires more detailed studies in the future. This findings suggest that there are possibly new factors or mechanisms having potence to interfere with there platelet aggregation responses.

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Vol.2, No.7, 713-721 (2010)
doi:10.4236/health.2010.27109

Health

The effect of past and present lifestyle, nutrition habits, and gender on bone mineral density

Masakatsu Nakada[1]*, Shinichi Demura[2]

[1]National Defense Academy Hashirimizu, Yokosuka, Japan;
*Corresponding Author: nakada@nda.ac.jp
[2]Graduate school of Natural Science & Technology, Kanazawa University Kakuma, Kanazawa, Japan

Received 2 March 2010; revised 14 March 2010; accepted 16 March 2010.

ABSTRACT

This study aimed to examine the gender and age differences of the quantitative osteo-sono assessment index (OSI) and the effects of the past and present lifestyle and nutrition habits on OSI in adult males and females from 20 to 70 years of age. The subjects were 155 males (20-79 years) and 399 females (20-78 years). The bone mass was estimated by the right-calcaneal OSI using an ultrasonic transmission method with an AOS-100 device (ALOKA). The frequency of tests for OSI in women tended to increase rapidly in the 50-70 age group requiring close examination or guidance. In 50-70 year-old females, the proportion of dairy products and vitamin D intake in the past (junior high school and high school days) was significantly lower in the group requiring close examination or guidance (OSI < 2.428) than in the normal group (OSI ≧ 2.428). That is, there was insufficient calcium intake (through dairy products) and vitamin D intake, which is instrumental in calcium absorption, (through fish, chicken eggs, and fungi) during puberty, when bone mass increases with skeletal growth. In conclusion, the number in the group requiring close examination or guidance was high for 50-70 year-old males and females. The OSI decreases rapidly in females after their 50s and the number in the group requiring close examination or guidance increased rapidly.

Keywords: Lifestyle Habit; Nutrition Habit; Ultrasound; Osteo-Sono Assessment Index; Gender

1. INTRODUCTION

Osteoporosis in elderly people markedly results in a decrease in activities of daily living (ADL) and quality of life (QOL) [1-4]. Aging is an important factor which affects bone mass and bone mineral density (BMD) [5]. BMD reaches its peak level from puberty to the time a person reaches their 20s and is maintained until their 40s, and then begins to decrease [2]. Nakata *et al.* [6] reported that because it is difficult to prevent osteoporosis in middle age with low peak bone mass, it is important to acquire basic eating and exercise habits during young adult age. Preventing a decrease of the peak bone mass through proper nutrition, exercise, sun bathing, etc. is very effective for preventing osteoporosis in old age [5]. It has been reported that eating habits in addition to exercise habits greatly affect bone formation [3,7]. Kim *et al.* [8] found that the loss of bone mineral content and bone mass with age differs by gender. Hence, the effect of lifestyle on BMD and bone mass after acquiring the peak bone mass should be studied according to gender and by using people of a wide age range.

Calcium ingestion during puberty markedly increases bone mass and may be an important factor in determining peak bone mass [2]. Bone mass in females decreases by about 3 percent a year with age after menopause [9]. Hence, increasing peak bone mass as much as possible during puberty is very important, and also the examination of relationships between BMD after adolescence and past (puberty) lifestyle habits will be essential.

Nakada *et al.* [10] confirmed that the effect of past and present lifestyle habits and nutrition on calcaneal quantitative osteo-sono index (OSI) in pre- and post-menopausal females. This study aimed to examine the OSI differences among different gender and age groups, and the effect of past and present lifestyle and nutrition habits on OSI in people from 20 to 70 years of age.

2. METHODS

2.1. Subjects

The subjects were 155 males (20-79 years of age) and

399 females (20-78 years of age). Written informed consent was obtained from all subjects after a full explanation of the experimental purpose and protocol.

2.2. Measurement of Bone Mineral Density and Setting of Osteo-Sono Assessment Index Group

The BMD was estimated by the right-calcaneus using an ultrasonic transmission method with an AOS-100 device (ALOKA). The calcaneal osteo-sono assesment was used osteo-sono assesment index (OSI: TI × SOS^2) by calculating the speed of sound (SOS) of an ultrasonic transmission in the calcaneus and transmission index (TI) referring to the report of Ishiguro *et al.* [11].

The Japan Osteoporosis Foundation [2] classified females into a close examination group (OSI < 80% of an average OSI = 2.158), a guidance required group (2.158 ≦ OSI < 90% of an average OSI = 2.428) and a normal group (OSI ≧2.428) based on an average OSI (OSI = 2.698) of females between 20 and 44 years old by osteo sono assessment criteria. In this study, we combined the former 2 groups considering a sample size of each age level and compared the close examination and guidance required groups (OSI < 2.428) and the normal group (OSI ≧ 2.428). The appropriate criteria of OSI in males has not been reported. Hence, males were classified into a close examination and guidance required group (OSI < 90%) and a normal group (OSI ≧ 90%) based on an average OSI of people between 20 and 44 years old in reference to the females' assessment criteria in Japan Osteoporosis Foundation [2].

2.3. Lifestyle Habits and Nutrition Questionnaire

Lifestyle habits and nutrition were evaluated by questionnaires. The survey was carried out just before the measurement of OSI. Naka *et al.* [5] selected menopause, habitual milk intake, intensity of physical exercise, and awareness of eating habits and physical activity as lifestyle items. Tomita selected breakfast habits, milk and dairy products, fish and shellfish, meat products etc. in present and childhood (about 6-15 yr) as eating habit items. Elgán *et al.* [12] selected 10 items (dietary habits (i.e. sugar, fat, fiber, and fruit/vegetables), physical activity, smoking habits, alcohol consumption, time spent outdoors etc.) as lifestyle items. The Japan Osteoporosis Foundation [2] selected alcohol, tobacco, coffee, milk, dairy products, fish, meat, soy products, green and yellow vegetables, and natto as meal and articles of taste items for the interview sheet as examples of osteoporosis prevention. Referring to the above, this study selected the following 9 items to investigate the present eating habits: 1) sleeping time, 2) frequency of alcohol consumption, 3) smoking habits, 4) skipping meals, 5) intake of dairy products (milk, cheese, yogurt, etc.), 6) intake of calcium supplements, 7) intake of vitamin D (fish, chicken egg, fungi), 8) intake of instant food (instant noodles, instant coffee, etc.), and 9) frequency of sun bathing. And, as the past (junior high school and high school days) states, 1) sleeping time, 2) skipping meals, 3) intake of dairy products, 4) intake of vitamin D, and 5) intake of instant food among the above 5 items were surveyed.

2.4. Data Analysis

Two–way (gender × age) ANOVA was used to examine the age and gender differences of OSI. When a significant difference was found, multiple comparisons were performed by Bonferroni's method. In both males and females, cross tabulations by 20-40 year-olds and 50-70 year-olds were made up. A χ^2 test was used to examine the proportion of OSI groups. In each OSI group, cross tabulations by the past and the present lifestyle and nutrition ingestion habits were made up and then a test of independence was performed. When a significant difference was found, residual analysis was used. A probability level of 0.05 was indicative of statistical significance.

3. RESULTS

Figure 1 shows the result of a two-way ANOVA (age × gender). A significant interaction effect was found. The results of multiple comparisons showed gender differences, males having a higher OSI, except for 30 year-olds. In males, the OSI of 20 year-olds was significantly higher than that of 30-70 year-olds. In females, the OSI of 20-40 year-olds was significantly higher than that of 50-70 year-olds.

Table 1 shows the result of χ^2 test (age × gender) in each OSI group. The frequency of the group requiring

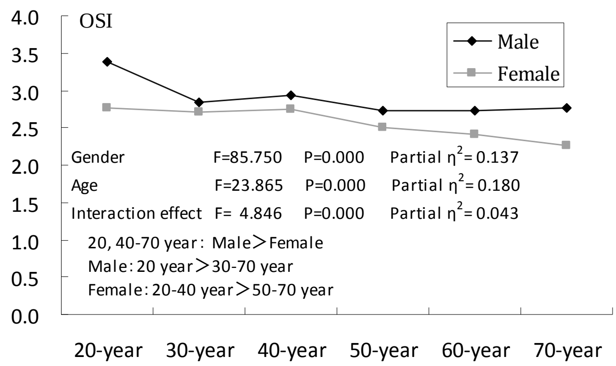

Figure 1. Result of two-way ANOVA (age x gender) in OSI.

Table 1. Result of χ^2 test (age × gender) in each OSI group.

gender	OSI group	20-40 year-olds	50-70 year-olds	χ^2	ρ	φ
Male	normal	59 (72%)	33 (45%)	11.451	0.000	0.285
	A close examination-guidance required	23 (28%)	40 (55%)			
Female	normal	155 (81%)	99 (48%)	46.21	0.000	0.349
	A close examination-guidance required	37 (19%)	108 (52%)			

close examination or guidance showed a significant difference in males and females. The Average OSI of 20-44 year-old males was 3.034 and 90% of that corresponds to 2.731. We used this value as the judgment criteria for the group requiring close examination or guidance, similar to the female group. The number of male subjects corresponding to less than OSI = 2.731 in males out of the 20-40 year-olds and 50-70 year-olds were 23 and 40, respectively.

Figure 2 shows the percentage of females in the group requiring close examination or guidance. The percentage tended to increase rapidly after 50 years of age (30-40 year-olds: about 1.5-1.7 times of 20 year-olds, 50 year-olds: about 3.4 times of 20 year-olds, 60 year-olds: about 4.3 times of 20 year-olds, and 70 year-olds: about 5.9 times of 20 year-olds).

Table 2 (20-40 years-old males) and **Table 3** (50-70 year-old males) show the cross tabulations of the frequency of OSI groups and the frequency of the present and past lifestyle and nutrition habits. A test of independence showed significant differences in the frequency in the intake of vitamin D in the 50-70 year-old males. However, the results of residual analysis showed no significant differences in any category. In the 20-40 year-old males, there were no significant differences in all present lifestyle and nutrition ingestion habit items.

Table 4 (20-40 year-old females) and **Table 5** (50-70 year-old females) show the cross tabulations of the frequency of OSI groups and the frequency of the present and past lifestyle and nutrition habits. In the 20-40 year-old females, a test of independence showed significant differences in sleeping time. However, the results of residual analysis showed no significant differences in any category. A test of independence showed significant differences in the intake of dairy products and vitamin D in the past in the 50-70 year-old females. The results of residual analysis showed significant differences in the intake of dairy products in the past; the normal group showed a higher proportion of subjects with a greater weekly intake (dairy products: z = 2.95 > 2.64, Vitamin D: z = 2.75 > 2.64, $p < 0.05$).

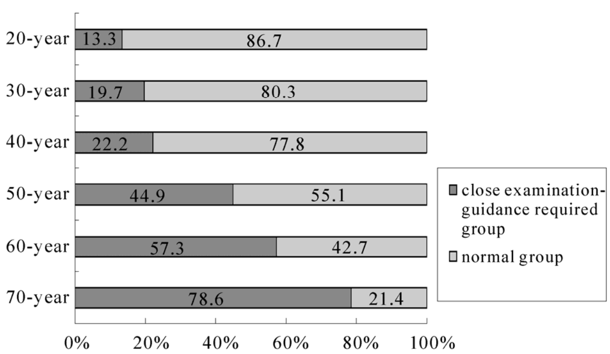

Figure 2. Frequency of OSI in the close examination-guidance required group in female.

4. DISCUSSION

The Japan Osteoporosis Foundation [2] reported that average OSI of 20 and 44 years old females (6096 people) was OSI = 2.698 ± 0.298. From the present result (OSI = 2.742 ± 0.350), it is considered that the OSI of females in this study was the standard. The gender differences of the OSI were found in all age groups, males being higher, except for 30 year-olds. In males, the age differences were found between the 20 year-old group and age groups after 30, and the OSI tended to remain the same level or to decrease slightly after their 30 s. However, in females, the OSI tended to be maintained in the 20-40 year-olds and to decrease sharply after their 50 s. Kim *et al.* [8] reported that bone mineral content in males decreased 0.3 kg per each decade from their 20 s to 40 s, kept almost the same level from their 40 s to their 60 s, and decreased 0.3 kg from their 60 s to their 70 s. In females, the bone mineral content changed little from their 20 s to their 40 s and decreased markedly from their 60 s to 70 s. It is thought that OSI is higher in males than in females and its decrease tendency with age differs largely by gender because of the sharp decrease observed in females beginning in their 50 s. And, the proportion in the group requiring close examination or guidance based on the judgment criteria by the Japan Osteoporosis Foundation [2] was higher in 50-70 year-olds than in 20-40 year-old males and females, but tended to increase sharply after 50 s (**Figure 2**) in females. This is thought to relate to the marked decrease of

Table 2. Present and past lifestyle and nutrition habits and the OSI of 20-40-year-olds males.

Sleeping time		Less than 6 hours	More than 6 hours-less than 7 hours	More than 7 hours-less than 8 hours	More than 8 hours	χ^2	ρ	φ
Present	Normal	12	33	12	1	2.817	0.421	0.19
	CEGR	4	14	3	2			
Past	Normal	9	20	18	6	0.981	0.806	0.11
	CEGR	2	9	9	2			
Alcohol intake		No	1-3 times a month	1-3 times a week	nearly every day			
Present	Normal	13	15	16	15	3.277	0.351	0.20
	CEGR	5	10	3	5			
Smoking		No	Have a habit	Quit				
Present	Normal	28	14	17		0.109	0.947	0.04
	CEGR	10	6	7				
Skip a meal		No	Breakfast	Lunch	Supper			
Present	Normal	45	13	0	1	6.452	0.092	0.28
	CEGR	21	1	1	0			
Past	Normal	45	12			0.568	0.451	0.09
	CEGR	19	3					
Intake of dairy products		No	1-3 times a week	4-7 times a week				
Present	Normal	10	25	24		0.476	0.788	0.08
	CEGR	5	8	10				
Past	Normal	7	23	27		0.371	0.831	0.07
	CEGR	4	9	10				
Intake of Ca supplement		No	Rarely	Continuous				
Present	Normal	40	14	5		3.691	0.158	0.21
	CEGR	20	3	0				
Intake of vitamin D		No	1-3 times a week	4-7 times a week				
Present	Normal	7	37	15		1.130	0.568	0.12
	CEGR	1	15	7				
Past	Normal	3	34	17		0.311	0.856	0.06
	CEGR	1	16	6				
Intake of instant food		No	1-3 times a month	More than once a week				
Present	Normal	9	20	30		2.425	0.297	0.17
	CEGR	2	12	9				
Past	Normal	3	30	24		0.39	0.823	0.07
	CEGR	2	11	10				
Sunbathing		No	1-3 times a week	More than 4 times a week				
Present	Normal	14	24	20		1.734	0.42	0.15
	CEGR	5	13	5				

Note: CEGR:close examination or guidance required group

Table 3. Present and past lifestyle and nutrition habits and the OSI of 50-70-year-olds males.

Sleeping time		Less than 6 hours	More than 6 hours-less than 7 hours	More than 7 hours-less than 8 hours	More than 8 hours	χ^2	ρ	φ
Present	Normal	6	14	11	1	3.316	0.345	0.21
	CEGR	9	13	13	6			
Past	Normal	1	11	17	1	2.89	0.409	0.21
	CEGR	2	14	13	4			
Alcohol intake		No	1-3 times a month	1-3 times a week	nearly every day			
Present	Normal	9	1	5	17	1.095	0.778	0.12
	CEGR	15	2	4	20			
Smoking		No	Have a habit	Quit				
Present	Normal	11	6	13		1.179	0.555	0.13
	CEGR	11	7	23				
Skip a meal		No	Breakfast	Lunch	Supper			
Present	Normal	25	3	1	0	2.272	0.321	0.18
	CEGR	39	2	0	0			
Past	Normal	21	4	1	0	1.801	0.406	0.17
	CEGR	33	4	0	0			
Intake of dairy products		No	1-3 times a month	4-7 times a week				
Present	Normal	5	13	14		0.579	0.749	0.09
	CEGR	9	14	18				
Past	Normal	11	12	7		4.078	0.13	0.25
	CEGR	21	13	3				
Intake of Ca supplement		No	Rarely	Continuous				
Present	Normal	27	3	2		0.595	0.743	0.09
	CEGR	34	4	1				
Intake of vitamin D		No	1-3 times a week	4-7 times a week				
Present	Normal	1	23	8		0.415	0.813	0.08
	CEGR	2	31	8				
Past	Normal	0 (–1.80)	20 (–1.20)	8 (2.56)		8.897	0.012*	0.37
	CEGR	4 (1.80)	31 (1.20)	2 (–2.56)				
Intake of instant food		No	1-3 times a month	More than once a week				
Present	Normal	6	18	8		2.797	0.247	0.20
	CEGR	13	15	12				
Past	Normal	14	10	5		0.139	0.933	0.05
	CEGR	16	14	6				
Sunbathing		No	1-3 times a week	More than 4 times a week				
Present	Normal	6	12	12		1.400	0.497	0.14
	CEGR	4	18	18				

Note: CEGR: close examination or guidance required group, *$P < 0.05$, Number shown in parentheses is the Z score of the residual analysis.

Table 4. Present and past lifestyle and nutrition habits and the OSI of 20-40-year-olds females.

Sleeping time		Less than 6 hours	More than 6 hours-less than 7 hours	More than 7 hours-less than 8 hours	More than 8 hours	χ^2	ρ	φ
Present	Normal	49 (0.54)	70 (–2.15)	35 (2.38)	1 (–1.11)	8.359	0.039*	0.21
	CEGR	10 (–0.54)	24 (2.15)	2 (–2.38)	1 (1.11)			
Past	Normal	21	50	39	9	7.405	0.060	0.22
	CEGR	1	11	11	6			
Alcohol intake		No	1-3 times a month	1-3 times a week	nearly every day			
Present	Normal	47	63	21	23	3.537	0.316	0.14
	CEGR	14	9	5	8			
Smoking		No	Have a habit	Quit				
Present	Normal	128	13	13		0.458	0.795	0.05
	CEGR	29	4	4				
Skip a meal		No	Breakfast	Lunch	Supper			
Present	Normal	125	17	3	4	2.393	0.495	0.11
	CEGR	29	6	0	0			
Past	Normal	110	30	0	0	0.113	0.737	0.03
	CEGR	26	6	0	0			
Intake of dairy products		No	1-3 times a month	4-7 times a week				
Present	Normal	15	62	78		0.699	0.705	0.06
	CEGR	2	16	19				
Past	Normal	16	58	72		2.592	0.274	0.12
	CEGR	2	18	13				
Intake of Ca supplement		No	Rarely	Continuous				
Present	Normal	113	29	12		1.483	0.476	0.09
	CEGR	30	6	1				
Intake of vitamin D		No	1-3 times a week	4-7 times a week				
Present	Normal	8	88	58		2.501	0.286	0.11
	CEGR	3	25	9				
Past	Normal	4	86	49		4.167	0.125	0.16
	CEGR	3	18	7				
Intake of instant food		No	1-3 times a month	More than once a week				
Present	Normal	28	56	69		5.915	0.052	0.18
	CEGR	2	11	24				
Past	Normal	22	76	45		0.060	0.970	0.02
	CEGR	4	16	9				
Sunbathing		No	1-3 times a week	More than 4 times a week				
Present	Normal	43	62	47		2.545	0.280	0.12
	CEGR	14	10	13				

Note: CEGR: close examination or guidance required group, $^*P < 0.05$, Number shown in parentheses is the Z score of the residual analysis.

Table 5. Present and past lifestyle and nutrition habits and the OSI of 50-70-year-olds females.

Sleeping time		Less than 6 hours	More than 6 hours-less than 7 hours	More than 7 hours-less than 8 hours	More than 8 hours	χ^2	ρ	φ
Present	Normal	19	49	26	2	2.401	0.493	0.11
	CEGR	26	50	26	6			
Past	Normal	5	25	36	11	0.506	0.917	0.06
	CEGR	4	26	32	13			
Alcohol intake		No	1-3 times a month	1-3 times a week	nearly every day			
Present	Normal	57	13	11	18	1.191	0.755	0.08
	CEGR	62	19	10	16			
Smoking		No	Have a habit	Quit				
Present	Normal	81	9	7		0.822	0.633	0.06
	CEGR	93	8	5				
Skip a meal		No	Breakfast	Lunch	Supper			
Present	Normal	87	5	0	1	3.444	0.328	0.13
	CEGR	95	3	3	1			
Past	Normal	66	12	0	0	2.224	0.329	0.12
	CEGR	72	10	2	0			
Intake of dairy products		No	1-3 times a month	4-7 times a week				
Present	Normal	3	30	65		3.592	0.116	0.13
	CEGR	7	41	57				
Past	Normal	14(–0.18)	44(–0.93)	27(2.95*)		9.751	0.008*	0.24
	CEGR	24(0.18)	50(0.93)	11(–2.95*)				
Intake of Ca supplement		No	Rarely	Continuous				
Present	Normal	73	11	15		0.803	0.669	0.06
	CEGR	73	16	15				
Intake of vitamin D		No	1-3 times a week	4-7 times a week				
Present	Normal	8	51	39		0.108	0.947	0.02
	CEGR	10	54	43				
Past	Normal	5(–1.38)	42(–1.82)	36(2.75*)		8.189	0.017*	0.22
	CEGR	10(1.38)	53(1.82)	19(–2.75*)				
Intake of instant food		No	1-3 times a month	More than once a week				
Present	Normal	35	30	31		0.727	0.695	0.06
	CEGR	34	32	40				
Past	Normal	30	39	14		0.372	0.830	0.05
	CEGR	33	35	13				
Sunbathing		No	1-3 times a week	More than 4 times a week				
Present	Normal	12	35	47		0.955	0.620	0.07
	CEGR	17	38	44				

Note: CEGR: close examination or guidance required group, *P < 0.05, Number shown in parentheses is the Z score of the residual analysis.

bone mass with a rapid decline of estrogen levels in postmenopausal females [13]. The OSI is generally higher in males than females. Average OSI of 20-44 years people also in this study showed a significant gender difference (males: OSI = 3.034 ± 0.396, females: OSI = 2.742 ± 0.350, t = 5.283, P = 0.000). The proper criteria has not been reported for males, so this study conveniently utilized the adult female criteria creation method developed by the Japan Osteoporosis Foundation [2]. It is considered that males' OSI level is higher and thus the criteria for the group requiring close examination or guidance differs between genders. Hence, from now, the OSI standard for males will need to be hastily created based on a large amount of data.

The group requiring close examination or guidance in 50-70 year-old females had a lower proportion of subjects with weekly intake (4-7 times a week) of dairy products and vitamin D (fish, chicken egg, fungi) in the past than the normal group. Tomita *et al.* [7] reported that, in the study of junior college dietetics students, the intake of milk, dairy products, and vegetables on a routine basis is useful to increase bone mass. Nakata *et al.* [6] reported that calcaneal OSI in women's junior college students was higher in the high milk intake group. The three year longitudinal study by Dawson-Hughes *et al.* [14] found that the proper intake of vitamin D in addition to calcium intake reduces the decline of BMD. It was reported also that inadequate metabolism of vitamin D decreases calcium absorption in both osteoporotics and elderly subjects [15]. From the present results, it is considered that the 50-70 year-old females belonging to the group requiring close examination or guidance were deficient in calcium intake through dairy products and intake of vitamin D which is very important for the absorption of calcium during puberty to increase bone mass with skeletal growth. In addition, the above suggests that it is important to have adequate calcium and vitamin D intake in puberty in addition to old age.

Meanwhile, males showed an insignificant relationship between OSI and items involving the present and past lifestyle and nutrition habits. Because they do not have a large physiological change as females when they experience menopause in middle age, the effect of the intake of calcium and vitamin D during puberty on maintenance of BMD and bone formation may be lower than in females. Many bone fractures that occur in elderly people are of the femoral neck and this fracture causes bedriddenness and disturbances of gait. Therefore preventing the cause, osteoporosis, is very important [2,5].

From now, it will be necessary to compare bone mineral density between young adults and the elderly longitudinally, and to examine the combined effect of the past and present lifestyle and exercise habits on BMD.

In summary, the OSI is higher in males than females and it is higher in 20 year-olds than 30-70 year-olds in males and is higher in 20-40 year-olds than 50-70 year-olds in females. The proportion of the group requiring close examination or guidance is high in 50-70 year-olds, particularly in females with a rapid increase after their 50s. The 50-70 year-old females in the group requiring close examination or guidance were deficient in calcium intake through dairy products in addition to the intake of vitamin D, which is important for the absorption of calcium during puberty when bone mass increases with skeletal growth. The intake of calcium and vitamin D during puberty may be very important to prevent the decrease of bone mineral density in old age.

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Vol.2, No.7, 722-730 (2010)
doi:10.4236/health.2010.27110

Health

Influence of poloxamer 407 on fractional and subfractional composition of serum lipoproteins of mice

Tatyana A. Korolenko[1]*, Fedor V. Tuzikov[2,3], Thomas P. Johnston[4], Natalia A. Tuzikova[2,3], Elena E. Filjushina[1], Viktoriya M. Loginova[1], Natalia G. Savchenko[1]

[1]Institute of Physiology, Siberian Branch of Russian Academy of Medical Sciences, Novosibirsk, Russia;
[*]Corresponding Author: t.a.korolenko@physiol.ru
[2]Boreskov Institute of Catalysis, Siberian Branch of Russian Academy of Sciences, Novosibirsk, Russia
[3]Novosibirsk State University, Novosibirsk, Russia
[4]University of Missouri-Kansas City, Kansas City, USA

Received 4 March 2010; revised 22 March 2010; accepted 25 March 2010.

ABSTRACT

Using a novel small-angle X-ray scattering (SAXS) method for determination of fractional and *sub*fractional composition of lipoproteins (LPs), a significant elevation of *total* cholesterol-lipoproteins (C-LP) and, especially, total triglyceride-lipoproteins (TG-LP), was shown in this work. Among the LP fractions, poloxamer 407 was shown to significantly increase proatherogenic total C-LDL, TG-LDL and, especially, their precursors C-VLDL and TG-VLDL, while only exhibiting a moderate increase in the antiatherogenic C-HDL and TG-HDL fractions. With regard to the VLDL *sub*fractions, significant elevations were observed in both *sub*fractions studied; namely, C-VLDL$_{1-2}$ and C-VLDL$_{3-5}$. Similar changes were noted in the TG-VLDL$_{1-2}$ and TG- VLDL$_{3-5}$ *sub*fractions. The C-IDL and TG-IDL *sub*fractions were increased significantly (~20- to 30-fold), while the C-LDL$_{1-3}$ subfraction was moderately (~3- to 5-fold) increased at 48 hrs and at day 4. In the moderately elevated (~2- to 4-fold) anti-atherogenic HDL fraction, the C-HDL$_2$ *sub*fraction was increased more significantly (~4-fold) compared to the C-HDL$_3$ subfraction; however, both C-HDL *sub*fractions returned to baseline by day 4. The elevation in the TG-HDL$_2$ *sub*fraction was observed only at 24 hrs. Mouse models of hyperlipidemia and atherosclerosis are useful to evaluate the role of "individual" LPs, as well as their fractions and subfractions, in hyperlipidemia and the genesis of atherosclerosis.

Keywords: Poloxamer P-407; Dyslipidemia; Serum Lipoprotein Fractions and Subfractions

1. INTRODUCTION

Changes of different classes of circulating lipoproteins are the important indicies of lipid metabolism in physiology and pathology; lipoproteins have been shown to also play a regulatory role *in vivo* [1,2]. The main lipoprotein classes consist of pro-atherogenic low-density lipoproteins (LDL), very-low-density lipoproteins (VLDL), and anti-atherogenic high density lipoproteins (HDL), and are widely used as common lipid biomarkers in atherosclerosis [1]. However, the biological role of *sub*classes of lipoproteins is still under investigation. The regulatory role of lipoproteins has been shown to relate to many intracellular processes, primarily to plasma membrane permeability and fluidity as the result of changes in the concentration of plasma membrane cholesterol, with subsequent modifications of receptors and transmembrane proteins (transmitters). In this process, the role of total HDL and HDL subfractions have especially high importance in connection with their unique capacity to accept cholesterol from the plasma membrane and transport them to other classes of lipoproteins.

Hyperlipidemia is one of the main risk factors in the development of cardiovascular and cerebrovascular diseases common in contemporary society. In addition to the elevation of plasma LDL-cholesterol and VLDL-cholesterol, hypertriglyceridemia is suggested to be an additional independent risk factor for atherosclerosis and coronary heart disease [3]. According to recent data, atherosclerosis is not only a metabolic lipid disease, but has also been considered to result from inflammation (chronic inflammatory disease). Statins have been reported to not only lower LDL by inhibiting the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, but also by reducing inflammation to endothelial cells.

For the prevention of atherosclerosis, it is important to study the mechanisms of the *early* effects of hyperlipidemia on different cell types involved in the pathogenesis of atherosclerosis. Therefore, it is necessary to know the types of lipoproteins involved in hyperlipidemia at the early stages of atherosclerosis. It is well known that during the process of atherosclerosis, foam cells initially trap the oxidized LDL molecules *via* scavenger receptors. The oxidized LDL molecule is digested and transformed, and is then presented to T lymphocytes, initiating the classic immunological reaction, and subsequently stimulates the inflammatory response. Formation of lipid-laden cells (mainly smooth muscle cells and macrophages) is followed by increased secretion of pro-inflammatory cytokines (like IL-6) and other factors (several types of matrix metalloproteases), which promote inflammation. Statin treatment has a pleiotropic protective effect, not only by inhibiting HMG-CoA reductase, but also by exerting its anti-inflammatory action.

The changes of serum lipoprotein levels responsible for pro- and anti-atherogenic action have been studied earlier [2]. However, recently, with help of new methods for characterizing different lipoprotein fractions and *subfractions*, some new data have been obtained on their role in the pathogenesis of atherosclerosis [4]. For this reason, the investigation of experimental models of hyperlipidemia is useful.

Poloxamer 407 is a block copolymer comprised of polyoxyethylene and polyoxypropylene units, which is known for its biocompatibility and potential to deliver different medications for a variety of disease states [5,6]. Following acute administration, poloxamer 407 was shown to induce significant hyperlipidemia, a model which has been used for testing several hypolipidemic drugs (statins, fibrates, and nicotinic acid) [7-11]. With chronic administration of poloxamer 407 (4 months) to mice, fibrofatty aortic lesions are developed, which are similar in size and number to those observed in classic diet-induced mouse models of atherogenesis [12,13].

Hyperlipidemia in acute poloxamer 407-induced administration to rodents was characterized by a dramatic elevation of both plasma cholesterol and triglycerides, which is usually not observed in patients with atherosclerosis. In the poloxamer 407 mouse model of atherosclerosis, the mechanism of cholesterol and TG elevation is associated with inhibition of cholesterol 7α-hydroxylase and lipoprotein lipase, respectively [13], and not dependent on PPARα [14]. It was also shown that a single injection of poloxamer 407 administration to mice caused hypercholesterolemia by inducing transient cholesterolgenesis and down-regulating low-density lipoprotein receptor expression [6]. The mechanism of elevation of different classes and, especially *subfractions* of lipoproteins with pro- and anti-atherogenic effects, is still not known.

Small-angle X-ray scattering (SAXS) is a small-angle scattering technique where the elastic scattering of X-rays (wavelength 0.1 to 0.2 nm) by a sample, which has inhomogeneities in the nanometer range, is recorded at very low angles. This angular range contains information about the shape and size of macromolecules, characteristic distances of partially ordered materials, pore sizes, and other data. SAXS is capable of delivering structural information of macromolecules between 5 and 25 nm, of repeat distances in partially ordered systems of up to 150 nm. SAXS is used in the characterization of various materials. In the case of biologic macromolecules, such as proteins, the advantage of SAXS, over crystallography, is that a crystalline sample is not needed. The materials can be solid or liquid and they can contain solid, liquid, or gaseous domains (so-called particles) of the same or another material in any combination. SAXS is accurate, non-destructive, and usually requires only a minimum of sample preparation.

The aim of the present investigation was to evaluate the lipoprotein-cholesterol and lipoprotein-triglyceride fractions and subfractions in hyperlipidemia induced by a single dose of poloxamer 407 to mice. Our overarching goal was to quantify the changes in the serum lipoprotein levels after poloxamer 407 treatment by utilizing a novel technique; specifically, small-angle X-ray scattering (SAXS).

2. MATERIALS AND METHODS

2.1. Materials

Male CBA/C57BL mice (breeding station of the Institute of Cytology and Genetics, Russian Academy of Sciences, Novosibirsk, Russia) having a body mass of 20-25 g were used. Poloxamer P-407 (Pluronic F-127, Sigma) was administered to mice, as a single, i.p. injection, in a dose of 1000 mg/kg. The animals were decapitated at 3, 24, 48 hrs, and then at 4, 5, 7, 12, 14 days after a single dose of poloxamer 407. All experiments followed the official “Rules for the work involving experimental animals” and “Ethical Committee Recommendations of working with laboratory animals”.

Serum was obtained after centrifugation of blood samples at 3000 × g for 15 min at 4°C (Eppendorf Centrifuge 5415R, Germany) and stored at –70°C until analysis of total cholesterol (C), triglycerides (TG) of lipoproteins (LP): C-LP, TG-LP, their fractions, and subfractions.

2.2. Small-Angle X-Ray Scattering (SAXS)

According to Otvos *et al.* (2002) [15], LP fractions were divided into the following 4 main classes: high density LP (HDL), low-density LP (LDL), very-low-density LP (VLDL), and chylomicrons (the last ones were not determined by the method used) or 7 subfractions: HDL_3, HDL_2, LDL, Intermediate-Density LP–IDL, $VLDL_{3-5}$, $VLDL_{1-2}$, chylomicrons. Interval borders of fractions and subfractions (according to scale of sizes, r_o) were the same as was indicated by Otvos (2002) [15].

A novel method for determination of fractional composition of LPs using the small-angle X-ray scattering approach (SAXS) was used [4]. This method is inexpensive, quick, and capable of determining the relative content of different LP fractions, both as a size distribution of various LP particles and as absolute units of the total concentration of lipid in LP fractions. At the same time, clinical diagnostic laboratories usually determine the concentrations of individual lipids in LP fractions, mainly TG and free cholesterol (FC), and often the total concentration of TG, FC and high density lipoproteins (HDL) free cholesterol.

Measurement of fractional and subfractional composition of serum LP fractions and subfractions.was provided according to a method described earlier [4]. SAXS roentgenograms were obtained using a Siemens diffractometer (Germany) by the method of step-by-step scanning with use of a goniometer and X-ray scintillation detector. Small-angle roentgenograms were measured in the angular range: $h = 0.013 \div 0.22$ A^{-1}, where $h = 4\pi \cdot \sin(\theta)/\lambda$; 2θ-scattering angle. A special thermostatted (20°C) quartz capillary cuvette (0.6 mm in diameter), having a wall thickness of 0.01 mm was used. The radiation wavelength (λ) was 1.54 E. The small-angle X-ray roentgenograms were corrected by taking into account background scattering, adsorption, and collimation, after which the X-ray data became smoothed. The first step of mathematical processing of the SAXS data and computation checks of functions for size distribution of spherical particles were executed using a special computer program and algorithms described earlier, and also by use of optimization programs [4].

The results are reported as the mean ± standard deviation of at least 3 different experiments for each sample analyzed. The differences between samples were analyzed by the Student's *t*-test, and a $P \le 0.05$ was considered statistically significant.

2.3. Morphological Study of Liver

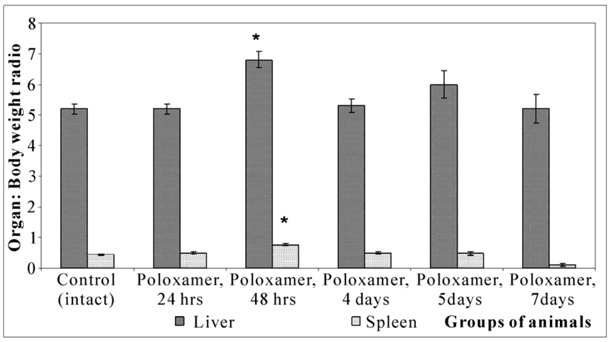
For morphometrical study of liver tissue, samples were first fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde on 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide solution, and then embedded in an Epon-Araldit mixture. Semithin, 1 micron tissue sections were obtained using the Ultramicrotome LKB-8800-V (Bromma, Vallingby, Sweden), stained with toludine blue and examined under a STAR Zeiss light microscope (Germany). The numeric density of macrophage cells was calculated as the number of cells per 1 $мм^2$ of tissue at a final magnification of 640. Not less than 50 fields of view were studied for every series of experiments. The cell measurements were made with the Motic Images 2000 Program. Ultrastructural changes of liver cells were investigated with an electron microscope, JEM 1400 (Jeol, Japan). Quantitative data were then processed using the Statistica-4 program, and the reliability of distinctions judged by the Student's *t*-test.

3. RESULTS

The relative weight of liver and spleen increased 48 hrs after poloxamer 407 administration (when the most significant hyperlipidemia was noted) (**Figure 1**). In peripheral blood, there was an increased number of monocytes observed at day 12 (**Figure 2**).

3.1. Morphological Studies

Light and electron microscopic studies demonstrated that liver sinusoids were considerably extended 1 day after poloxamer 407 administration, and that the numeric density of macrophages was significantly increased (almost twice) (**Table 1**). The heterogeneity of macrophage dimensions was strongly increased. Very large cells, with



X-axis: time after the single administration of poloxamer 407 (1000 mg/kg) to mice

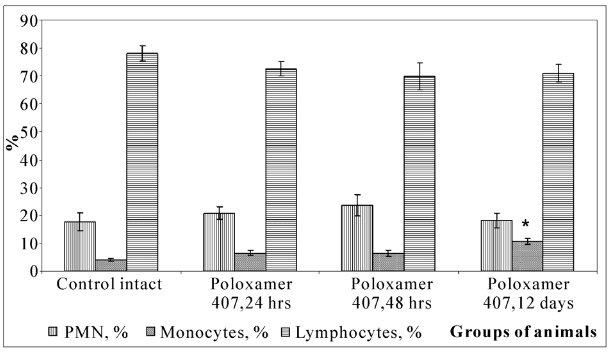
Y-axis: Organ: Body weight ratio of liver and spleen, %.

The relative weight was expressed as the organ: body weight ratio

Control group n = 26, poloxamer-treated groups the mean± SEM represent a minimum of 7 mice at each time point.

The data are shown as mean ± SEM (*p < 0.01 vs control)

Figure 1. Relative liver and spleen weight of mice during single administration of poloxamer 407 (1000 mg/kg) to mice.



X-axis: Time after poloxamer 407 administration to mice.
Y-axis: The number of PMN, monocytes and lymphocytes is expressed as % from the total amount of leukocytes.
Poloxamer was administered i.p. as a single dose (1000 mg/kg).
The number of animals in each group is 5.
The data are shown as mean ± SEM (*p < 0.05 vs control).

Figure 2. Influence of poloxamer 407 on leukocytes composition of the peripheral blood of mice (%).

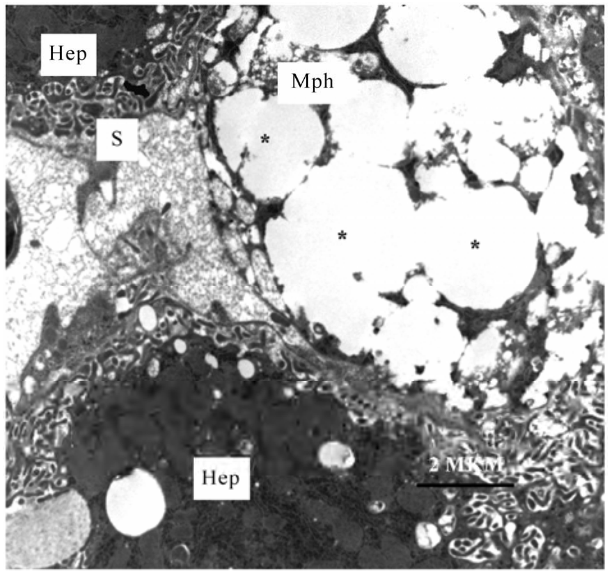
Table 1. The numerical density of liver macrophages during single administration of poloxamer 407 to mice.

Groups, the number of mice	The numeric density of liver macrophages, per mm^2
1. Control (intact) (5)	1277.4 ± 36.86
2. Poloxamer, 24 hrs (5)	2002.6 ± 36.58 P < 0.01
3. Poloxamer, 5 days after (5)	844.6 ± 28.74 P < 0.01

The data are shown as mean ± SEM.
The number of mice is in the parentheses.

a cross-sectional area more than 90-100 square microns, were observed among macrophages, which comprised about 40% of the macrophage population. These macrophages were filled with granular material, so their cytoplasm had a foamy appearance (**Figure 3**).

Five days after poloxamer 407 administration, morphometric data for the liver demonstrated that the specific numerical density of macrophages was decreased, not only in comparison with the previous value, but in relation to the controls as well (**Table 1**). As before, large macrophages with a "foamy" cytoplasm containing electron light (transparent) material (possibly, of lipid origin) were observed, but the fraction of these cells was considerably reduced compared to the number of these cells in the previous experiment (**Table 1**). The cytoplasm of hepatocytes in most cases was loose and mostly crumbly, with several large vacuoles, and their size was moderately increased.



Electron micrograph of liver cells. In sinusoid lumen a large liver macrophage with foamy cytoplasm, filled by numerous electron light granules.
Denotes: Mph–macrophage; Hep–hepatocyte; S-sinusoid
*-electron light granules of macrophages
Poloxamer was administered i.p. as a single dose (1000 mg/kg).
Study was performed at the 1st day after administration of a single dose of poloxamer 407.

Figure 3. Influence of poloxamer 407 on ultrastructure of liver cells.

3.2. Effect of Poloxamer 407 on Fractional and Subfractional Composition of Serum lipoproteins

3.2.1. Lipoprotein-Cholesterol (C-LP)

Compared to intact (Control) mice, there was a significant (~6-fold) increase in the serum *total* C-LP at 24 and 48 hrs, as well as day 4, in mice which were administered poloxamer 407 (**Table 2**). As shown in Table 2, among the total C-LP, the greatest increase (~50-fold) was observed with the pro-atherogenic C-VLDL fraction (both C-$VLDL_{1-2}$ and C-$VLDL_{3-5}$ subfractions), as well as with intermediate density lipoproteins (C-IDL) (~20-fold). However, it should be noted that serum concentrations of each fraction begin to decrease by day 4. The C-LDL concentration increased about twice, relative to controls, and continued to remain elevated at day 4. C-LDL_{1-3} was moderately (~3- to 5-fold) increased at 48 hrs and at day 4 (**Table 2**). The C-IDL and TG-IDL *sub*-fractions were increased significantly (~20-30-fold), while the C-LDL_{1-3} subfraction was moderately increased (~3- to 5-fold) at 48 hrs and at day 4.

Among anti-atherogenic fractions and subfractions, only a moderate increase of C-HDL was noted (~2- to

Table 2. Concentration of C-LP fractions and subfractions (mg/dl) in murine serum during administration of poloxamer 407 (1000 mg/kg).

LP	Control, intact (n = 10)	Poloxamer, 24 hrs (n = 10)	Poloxamer, 48 hrs (n = 8)	Poloxamer, 4 days (n = 5)
C-LP (All)	107.4 ± 8.84	662.9 ± 82.40***	599.7 ± 104.11**	727.2 ± 87.2*
C-VLDL	6.5 ± 1.85	345.8 ± 46.90***	271.3 ± 73.02**	134.4 ± 46.23**
C-VLDL$_{1-2}$	0.3 ± 0.07	16.7 ± 2.13***	12.8 ± 4.70**	8.9 ± 4.11
C-VLDL$_{3-5}$	6.0 ± 1.71	329.1 ± 45.54***	258.5 ± 70.44**	125.5 ± 42.43**
C-LDL	43.1 ± 7.67	106.9 ± 24.15**	201.0 ± 34.38***	247.3 ± 31.52***
C-IDL	1.9 ± 1.61	40.3 ± 11.13**	62.6 ± 14.88***	34.9 ± 13.32*
C-LDL$_{1-3}$	41.2 ± 8.61	66.6 ± 25.54	146.1 ± 32.68**	212.4 ± 35.56**
C-HDL	57.9 ± 9.75	210.2 ± 48.36**	127.4 ± 41.93	45.4 ± 13.30
C-HDL$_2$	43.1 ± 10.02	166.5 ± 38.70**	91.9 ± 47.31	16.9 ± 8.55
C-HDL$_3$	14.7 ± 3.55	43.7 ± 20.21	35.5 ± 8.94 *	28.5 ± 9.23

The data are shown as mean ± SEM (*p< 0.05; **p < 0.01; ***p < 0.001 vs control)

The number of animals is in the parentheses.

Abbreviations: C-LP–cholesterol of lipoproteins; C-HDL–cholesterol of high density lipoproteins; C-LDL–cholesterol of low density lipoproteins; C-IDL–cholesterol of intermediate density lipoproteins; C-VLDL–cholesterol of very low density lipoproteins.

4-fold), with a return to baseline by day 4 (primarily due to a decrease in the concentration of the C-HDL$_2$ subfraction), while the C-HDL$_3$ subfraction showed a moderate increase (**Table 2**).

3.2.2. Lipoproteins-TG (TG-LP)

In general, the total amount of TG-LP increased more significantly as compared to C-LP (~20-fold), with a tendency to return to normal levels by day 4 (**Tables 2** and **3**). TG-VLDL "behavior" was similar to C-VLDL (increased 20- to 40-fold), and both *sub*fractions (TG-VLDL$_{1-2}$ and TG-VLDL$_{3-5}$) were responsible for the elevation of the TG-VLDL fraction (**Tables 2** and **3**). Similar to C-LDL, an increase in TG-LDL was observed (~6- to 8-fold). The level of C-IDL (**Table 2**) and TG-IDL (**Table 3**) was shown to be increased (~18- to 28-fold, respectively) with a significant difference at 24, 48 hrs, and on day 4, as compared to the controls (**Tables 2** and **3**). A moderate elevation was also observed for C-HDL and TG-HDL (~2- to 3-fold) by 24 hrs, but serum levels were not significantly greater than controls from 24 hrs onward (**Tables 2** and **3**). The TG-HDL$_2$ subfraction was elevated only at 24 hrs after poloxamer 407 administration when compared to controls (**Table 3**). There were no significant changes of the TG-HDL$_3$ *sub*fraction (**Table 3**).

4. DISCUSSION

Atherosclerosis is a complex disorder of lipid metabolism and a chronic inflammatory disease [16]. Changes in lipoprotein metabolism and hyperlipidemia play an important role in the progression of atherosclerosis. The initial period of atherosclerosis development and the interaction of lipoproteins with cells involved in the dislipidemic state merits further investigation. Mouse models of hyperlipidemia and atherosclerosis are useful tools with which to study the role of "individual" lipoproteins of different classes.

Lipoproteins are divided into 4 main fractions (classes)—HDL, LDL, VLDL, and chylomicrons, or 7 *sub*fractions (HDL$_3$, HDL$_2$, LDL, IDL, VLDL$_{3-5}$, VLDL$_{1-2}$, chylomicrons), or 16 *sub/sub*fractions [15,17,18]. Most serum cholesterol, about 60-70% of total serum cholesterol, is known to be contained in LDL-C as the major atherogenic lipoprotein fraction [1]. During the last decade, several new methods, including the analytical ultracentrifugal micromethod, were developed for identification of atherogenic LDL, and their subclasses, without their preparative isolation [19].

At the same time, it is important to investigate other lipoprotein fractions—namely, VLDL and HDL, as well as their *sub*fractions. VLDL, which is a precursor of LDL, and some forms of VLDL, is atherogenic, especially their remnant forms. VLDL contains most of the serum triglycerides, their role, of which, is now thought to be an independent and important risk factor in atherosclerosis [20]. HDL is known as an antiatherogenic fraction of lipoproteins [21]. With the assistance of the new physicochemical SAXS method, it is possible to simultaneously

Table 3. Concentration of TG-LP fractions and subfractions (mg/dl) in murine serum during administration of poloxamer 407 (1000 mg/kg).

LP fractions, Subfractions	Control, intact (n = 10)	Poloxamer, 24 hrs (n = 10)	Poloxamer, 48 hrs (n = 8)	Poloxamer, 4 days (n = 5)
TG-LP (All)	54.5 ± 6.57	1107.4 ± 148.48***	902.2 ± 223.69**	517.3 ± 152.67**
TG-VLDL	19.5 ± 4.85	952.1 ± 129.69***	735.2 ± 198.61**	383.0 ± 140.44**
TG-VLDL$_{1-2}$	3.1 ± 0.61	148.7 ± 26.59***	103.9 ± 36.43**	80.1 ± 41.67
TG-VLDL$_{3-5}$	16.0 ± 4.30	803.4 ± 114.37***	631.3 ± 175.49**	302.9 ± 101.23**
TG-LDL	14.0 ± 1.85	80.7 ± 13.92**	120.7 ± 33.23**	117.8 ± 14.79***
TG-IDL	2.6 ± 2.15	53.8 ± 14.83**	83.4 ± 19.82**	46.6 ± 17.74*
TG-LDL$_{1-3}$	11.4 ± 2.31	24.6 ± 10.37	47.5 ± 11.14**	71.2 ± 15.19**
TG-HDL	21.2 ± 3.58	76.9 ± 17.67**	46.4 ± 15.21	16.5 ± 5.00
TG-HDL$_2$	15.9 ± 3.68	60.9 ± 14.31**	33.6 ± 17.17	6.4 ± 3.16
TG-HDL$_3$	5.3 ± 1.25	16.1 ± 7.38	12.8 ± 3.14	10.14 ± 3.39

The data are shown as mean ± SEM ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs control)

The number of animals is in the parentheses.

Abbreviations: TG-LP-triglycerides of lipoproteins; TG-HDL–triglycerides of high density lipoproteins; TG-LDL–triglycerides of low density lipoproteins; TG-IDL–triglycerides of intermediate density lipoproteins; TG-VLDL–triglycerides of very low density lipoproteins.

investigate all three major classes of lipoproteins (VLDL, LDL, HDL) and all *sub*fractions, except chylomicrons (the particles are too large for this method of analysis) [4]. Moreover, it is known that fasting human plasma, as well as plasma of mice 12-14 hrs after feeding, contains negligible chylomicrons. The available physical-chemical data in the literature suggests the existence of a stable thermodynamic equilibrium between all lipoprotein types. This equilibrium is specific for each physiological state in the human and helps to stabilize the lipoprotein structure and assist with normal lipoprotein functioning. In the SAXS method, the dynamic equilibrium between different LP types was used as a physico-chemical basis, and a general model, which takes into account the relative amount of all LP types and their transitional forms (in terms of the relative size of LP particles and their relative concentrations) was developed [4].

As was shown in this work, the poloxamer 407-induced model of hyperlipidemia in mice was characterized by a significant elevation of both C-LP and TG-LP *total* fractions. Similar data were obtained by other investigators that studied the level of atherogenic and antiatherogenic lipoprotein fractions with other, more common methods [5]. It is necessary to mention that the concentration of pro-atherogenic LDL and VLDL fractions increased dramatically, as compared to a moderate increase in the level of HDL particles. This is probably one of the reasons why repeated administration of poloxamer 407 to mice successfully reproduces the atherosclerotic process in rodents, where atherosclerosis is difficult to develop due to the normally high concentration of antiatherogenic HDL. These data were supported by the observation that statin treatment in patients with cardiovascular diseases decreases the mortality by decreasing the C-LDL levels, thereby, exerting the protective lipid-lowering and also pleiotropic effects [2].

Elevated plasma concentration of C-LDL is considered a risk factor for atherosclerosis development. LDL, especially oxLDL particles, infiltrates the vascular wall and can be taken up by macrophages. This is followed by secretion of proinflammatory factors which are involved in the pathogenesis of atherosclerosis. HDL, like other fractions of lipoproteins (LDL and VLDL), are a heterogeneous group of lipoproteins; their core contains some free cholesterol, triglycerides, and cholesterol esters [21,22]. HDL particles are involved in reverse cholesterol transport, and their atheroprotective role may be connected with regulation of adhesion molecule expression and the prevention of oxidative modifications of LDL [16,21]. It was shown in this work that both *sub*fractions of C-HDL were only moderately increased in poloxamer 407-treated mice and returned to normal levels by day 4 following poloxamer administration. Similar changes were observed in the TG-HDL fraction and *sub*fractions.

In general, there are many factors influencing serum concentration of lipoproteins (biosynthesis, uptake by endocytosis, secretion, and degradation). The liver is the main source of VLDL. LDL catabolism occurs primarily in the liver and in most extrahepatic tissues [2]. Different types of cells have been shown to have LDL receptors on the plasma membrane [6]. Catabolism of LDL has been shown to occur inside of lysosomes [23,24]. Metabolism of HDL is closely related to homeostasis of lipids in the intact vertebrate, with lipoproteins residing in both the serum and in tissues [2].

Poloxamer 407 appears to act as a general lipase inhibitor. Mammalian lipases include three main lipase members; namely, lipoprotein lipase, hepatic lipase, and endothelial lipase, acting *in vitro* and *in vivo* on lipoproteins [23]. Lipoprotein lipase has a high triglyceride lipase activity, while endothelial lipase exerts phospholipase activity. Endothelial lipase is able to hydrolyze lipids from HDL, while hepatic lipase exerts functional activity on all classes of lipoproteins [23]. Lipoprotein lipase (EC 3.1.1.34) (LPL) is a key enzyme in lipoprotein metabolism. LPL belongs to the class of enzymes known as hydrolases, which catalyze the degradation of glycerides, including triglycerides. Poloxamer 407 (as well as the nonionic detergent Triton WR 1339) has been shown to inhibit LPL, and this effect has been suggested as playing a crucial role in disturbances of lipoprotein transport and a significant elevation in the serum levels of LP-TG.

There are some similarities in the models of poloxamer and Triton WR 1339-induced hyperlipidemia following a single intraperitoneal administration of each agent. Both models were characterized by *dramatic* increased serum concentrations of cholesterol and, especially triglycerides, in rats and mice. As mentioned above, the resultant hypertriglyceridemia occurs due to surfactant-mediated inhibition of LPL's enzymatic activity [25,26]. Moreover, Triton WR 1339 is a well-known lysosomotropic agent, accumulating inside of lysosomes of liver cells; specifically, hepatocytes and Kupffer cells [27]. In both species of rodents, Triton-induced hyperlipidemia occurred 24 hrs after detergent administration, and was followed by a dramatic increase in both lipoprotein cholesterol and, especially, of lipoprotein triglyceride concentrations [26,27]. We have recently shown significant increases in the concentration of atherogenic C-LDL, C-VLDL (due to an increase of the $VLDL_{3\text{-}5}$ subfraction), and IDL in mice, and even more profound elevations in rats (Korolenko *et al.*, submitted *In Press*). These hyperlipidemic animal models can be used to study the role of lipoproteins, especially lipoprotein triglycerides, (but also C-LDL and C-VLDL) in the pathogenesis of atherosclerosis, as well as for testing the efficacy of new hypolipidemic drugs. The poloxamer 407-induced mouse model of hyperlipidemia would appear to resemble a Type 2a/2b or 3 dislipidemia in humans.

Mice and rat models of hyperlipidemia are used more often now because of their convenience, the ability to genetically alter the animals to evaluate the effect of a particular gene on lipid metabolism, and because they are cost-effective. The poloxamer 407-induced mouse model of dyslipidemia and atherosclerosis reliably reproduces the hyperlipidemic state, and, if administered on a repetitive basis (or, if dosed continuously with the aid of an implanted osmotic pump), causes formation of fibrofatty aortic lesions in the same size and number as classic diet-induced or gene-knockout mouse models [28].

Poloxamer 407 has been used in several fields of medicine as a nano-carrier for different drugs for the treatment of several inflammatory diseases and tumors [29]. However, when used to induce dyslipidemia and atherosclerosis in C57BL/6 mice, it must be emphasized that these are supraphysiologic doses of P-407 that would never be utilized in any commercial formulation employing its sustained-release or reverse-thermal gelation properties. Additionally, most applications of poloxamer 407 in the pharmaceutical literature deal with non-parenteral routes of delivery, unlike the intraperitoneal administration of poloxamer 407 to intentionally induce dyslipidemia in mice [30-34].

In conclusion, we have identified changes in the lipid fractions and subfractions following a single dose of poloxamer 407 to mice, as well as characterized the relative spleen and liver weights, and the effects on blood leukocytes. Additionally, we have pioneered the use of SAXS as a method to determine the concentrations of the serum lipids and lipid subfractions. This new analytical approach can now be successfully employed to determine the concentrations of lipoprotein fractions, and their subfractions, in either mouse or human plasma samples for a more complete picture of the hyperlipidemic state.

5. ACKNOWLEDGEMENTS

This work was partially supported by integrated grant of Siberian Branch of the Russian Academy of Medical Sciences and Far East Division of Russian Academy of Sciences on natural immunomodulators study (2006-2008). Authors are grateful to engineer M.Tuzikov for help in Small-angle X-ray scattering, Dr. Monoszon A., Dr. Kisarova Ya., Dr. Sukhovershin R., Dr. K. Loktev for technical help during preparing of the manuscript.

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ABBREVIATIONS

C–cholesterol
TG–triglycerides
LP–lipoproteins
C (TG)-HDL–cholesterol (triglycerides) of high density lipoproteins
C (TG)-LDL–cholesterol (triglycerides) of low density lipoproteins
C (TG)-IDL–cholesterol (triglycerides) of intermediate density lipoproteins
C (TG)-VLDL–cholesterol (triglycerides) of very low density lipoproteins
SAXS-Small-angle X-ray scattering

Vol.2, No.7, 731-735 (2010)
doi:10.4236/health.2010.27111

Health

Prevalence, awareness, treatment and control of hypertension in a nigerian population

Obinna Ikechukwu Ekwunife*, Patrick Obinna Udeogaranya, Izuchukwu Loveth Nwatu

Department of Clinical Pharmacy and Pharmacy Management, University of Nigeria Nsukka; Department of Pharmacy, Enugu State Teaching Hospital Enugu State, Nigeria; *Corresponding Author: obinna.ekwunife@unn.edu.ng

Received 19 January 2010; revised 24 March 2010; accepted 26 March 2010.

ABSTRACT

Hypertension is a major public health problem. Due to paucity of data, the burden of hypertension in Nigeria might be underestimated. Estimating the prevalence of hypertension in populations of Nigeria would be useful in efforts to control hypertension and prevent its consequences. This survey aimed to assess the prevalence, detection, treatment and control of hypertension in Nsukka, a city located in South-Eastern Nigeria. Hypertension prevalence, awareness, treatment, and control (outcomes) were examined in 756 adult participants (364 men and 392 women) aged 18 years and above. Blood pressure (BP) of the participants was measured and they also answered a detailed questionnaire. Hypertension was defined as BP ≥ 140 for systolic BP and or ≥ 90 mm Hg for diastolic BP or being on antihypertensive therapy. Prevalence of hypertension was 21.1%. Men had higher prevalence of high BP compared to women. Systolic and diastolic BP increased with age in both men and women. Detection of high BP in participants with raised blood pressure was 40.3% and 24.7% for males and females respectively. Only 23.7% and 17.5% of males and females respectively with high BP were on antihypertensive treatment while 5.0% of males and 17.5% of females with hypertension were controlled. Prevalence of hypertension was comparable with other studies in Nigeria and Africa. The results showed a poor detection, treatment and control of hypertension. This underscores the need for comprehensive evaluation of the prevalence of hypertension and other cardiovascular diseases in Nigeria.

Keywords: Hypertension; Prevalence; Epidemiology; Nsukka

1. INTRODUCTION

Hypertension is a major public health problem. Worldwide, prevalence estimates for hypertension is about 1 billion individuals [1]. It causes about 7.1 million deaths per year [2] and 4.5% of the disease burden which translates to 64 million disability adjusted life years (DALYs) [3]. The relationship between blood pressure (BP) and risk of cardiovascular diseases events is continuous, consistent, and independent of other risk factors. The higher the BP, the greater is the chance of heart attack, heart failure, stroke, and kidney diseases [4].

The burden of non-communicable diseases (NCDs) such as hypertension is increasing in epidemic proportions in Africa. According to the World Health Report 2001**,** NCDs accounted for 22% of the total deaths in the region in the year 2000; cardiovascular diseases alone accounted for 9.2% of the total deaths, killing even more than malaria [2]. Major target-organ complications of hypertension, such as left ventricular hypertrophy [5], diastolic dysfunction [6], congestive heart failure [7], ischemic heart disease [8], stroke [9], and renal failure [10] have been established by various researchers in Nigeria.

Reducing the prevalence of hypertension would decrease mortality and disability in middle-aged and older persons and lead to a better quality of life. Reduction of hypertension prevalence could be achieved through risk factor prevention programmes as well as using low-cost management. However, in most countries of the African region, implementation of these approaches and programmes is hampered by dearth of data on the prevalence and control levels of hypertension. Scarcity of data is sometimes understood as non-existence of the problem [3]. There is paucity of hypertension prevalence in many populations of Nigeria. Thus, burden of hypertension in these populations might be underestimated and might leave the illness undiagnosed and untreated. Uncontrolled hypertension clearly places a substantial strain on health care delivery system. Estimating the prevalence of hypertension in populations of Nigeria would be useful in efforts to control hypertension and other NCDs. This survey aimed to assess the prevalence, detection, treatment and control of hypertension in Nsukka, a city located in South-Eastern Nigeria.

2. METHOD

2.1. Study Design and Population

This is a household survey with the objective of assessing prevalence, awareness and control of hypertension in Nsukka. Nsukka is a town located in South-Eastern Nigeria and has an estimated population of 117,086. Christianity is the main religion while farming, transportation and trading are the major commercial activities. The most prominent feature in Nsukka is the University of Nigeria Nsukka, which attracts people of different ethnic and linguistic group to the area. There are 19 public health facilities and over 20 private health facilities in Nsukka.

2.2. Ethical Consideration

All procedures were carried out according to a study protocol approved by the Local Ethics Committee of University of Nigeria Teaching Hospital Enugu. Objectives and nature of the study were explained to people that agreed to participate. Informed consent was orally obtained. The information about participant's identity was not included with the other data and only the principal investigator had access to this information. No reference to the participant's identity was made at any stage during data analysis.

2.3. Sampling and Sample Size Calculation

A mixture of cluster and systematic random technique was employed. Nsukka was grouped into 16 clusters based on geographical locations as established by a map designed by Nsukka Graduates Association. Six sections or clusters were randomly selected from the sixteen clusters using a random sampling technique. In each section, the first house in each street was identified, followed by systematic sampling of the next three houses. Using "Statcalc" function of EPI INFO (Version 6, Centre for Disease Control, USA), it was determined that a sample size of 400 was adequate to detect prevalence of hypertension of 10% to 40% with 5% precision and 95% confidence. However, a total of 800 persons were met in the exercise after covering the selected clusters.

2.4. BP Measurement and Interview Procedure

The data collection tool was a questionnaire. The survey was carried out from April to August of 2009. Participants that were included in the study were those from 18 years and above in each household identified. Those who took caffeine, smoked or did an exercise prior to the interview were excluded from the study. Participants were interviewed and blood pressure was measured at home. Data obtained were marital status, age, gender, educational status, estimated income per month, family history of hypertension, and co-morbidities. The interview was in English or in Igbo (the local dialect) for participants that could not understand English. Blood pressure was measured twice by the trained final year pharmacy students using mercury sphygmomanometers and stethoscopes (Kris-Alloy®, Wuxi Medical Instrument Factory, Wuxi City Jiangsu, China). Blood pressure was measured after they were in resting state for 10 minutes and in sitting position in the right arm place at the level of the heart. An appropriate-sized cuff (cuff bladder encircling at least 80% of the arm) was used to ensure accuracy [4]. High blood pressure was defined using the WHO/ISH criteria of SBP $\geq$ 140 mmHg and/or DBP $\geq$ 90 mmHg [11]. Prevalence of high BP was calculated as percentage of participants with SBP and DBP above WHO/ISH criteria, those that were known hypertensive and those on hypertension medication (s). Hypertension detection was defined as any prior diagnosis of hypertension made by a health professional among the population defined as having hypertension. Treatment of hypertension was defined as use of recognized anti-hypertensive medication among the population defined as having hypertension, whereas control of hypertension was defined as blood pressure of < 140/90 mmHg among the population defined as having hypertension.

2.5. Data Analysis

Mean levels of BP were reported by gender (male versus female) and age. Gender difference in blood pressure was conducted using 2-sample t-test while gender differences in detection, treatment and control rate was assessed using χ^2 test. All data analyses were conducted using SPSS 13.0 (Chicago, IL) software. Data were double checked by a staff of Clinical Pharmacy department, University of Nigeria Nsukka for consistency. A two-tailed significance level of 0.05 was used.

3. RESULTS

A total of 800 participants were encountered in their homes but only 756 participants agreed to participate in the study (94.5% response rate). Majority of those that declined to participate were afraid to be diagnosed of hypertension and would not want their blood pressure measured. The rest did not want to participate because of time the exercise will take. Three hundred and sixty four

(48.1%) were males while three hundred and ninety-two (51.9%) were females. Majority of the participants (about 60%) were married while the rest were single. Only a few of the participant (< 1%) were divorced. About 26% of the participants had a tertiary degree, 58% had up to secondary school training while 3% had no formal education. One half of the study population earned less than $ 100 in a month and men significantly earned more than women (T-test, $p < 0.001$). Details of the demographic characteristics of the study population are presented in **Table 1**.

Table 2 shows the details of clinical characteristics of the study population. Among the participants, 23.5% reported that they have a family history of hypertension. Mean systolic blood pressure in the study population was 129.8 ± 27.4 mmHg and mean diastolic blood pressure 85.1 ± 9.9 mmHg. Males had a significantly higher SBP compared to females –133.3 ± 14.3 mmHg vs 125.0 ± 14.6 mmHg respectively (T-test, $p < 0.001$). DBP was also significantly higher in males than in females –87.1 ± 9.3 mmHg vs 83.2 ± 10.2 mmHg respectively (T-test, $p < 0.001$). Prevalence of high blood pressure was 21.1%. Systolic and diastolic BP increased with age as shown in **Figure 1**.

Detection of high BP in participants with raised blood pressure was significantly higher in males (40.3%) compared to females (24.7%) ($\chi^2(1) = 4.2$, $p = 0.041$). Overall detection rate was 30%. Percentage of participants with high BP treated were 23.7% for males and 17.5% for males and females respectively. There was no significantly difference in treatment rate between men and women ($\chi^2(1) = 0.83$, $p = 0.36$). On the total, 21% of participants with high BP take medications. On control rate, 17.5% of the women were controlled while 5.0% of the men were controlled. There was significant gender difference in control rate ($\chi^2(1) = 6.73$, $p = 0.01$). Overall control rate of BP amongst hypertensive was 9% in the study population.

Table 1. Demographic characteristics of the study population (n = 756).

	Variable	Mean ± SD, frequencies (%) or median [Interquartile range]
1.	**Age (y)**	34.9 ± 13.9
	Gender	
2.	*Male*	364 (48.1)
	Female	392 (51.9)
	Marital status	
	Single	297 (38.4)
3.	*Married*	458 (59.2)
	Divorced	6 (0.8)
	Educational status	
	No formal education	23 (3.0)
4.	*Primary education*	87 (11.2)
	Secondary education	450 (58.1)
	Graduate	206 (26.1)
5.	**Average Individual Income ($/month)**	83.3 [83.3 – 250]

Table 2. Clinical characteristics of the study population.

	Variable	Mean ± SD or percentages (%)
1.	Family history of hypertension	23.5
2.	Systolic blood pressure (mm Hg)	129.8 ± 27.4
3.	Diastolic blood pressure (mm Hg)	85.1 ± 9.9
4.	Prevalence of BP	21.1

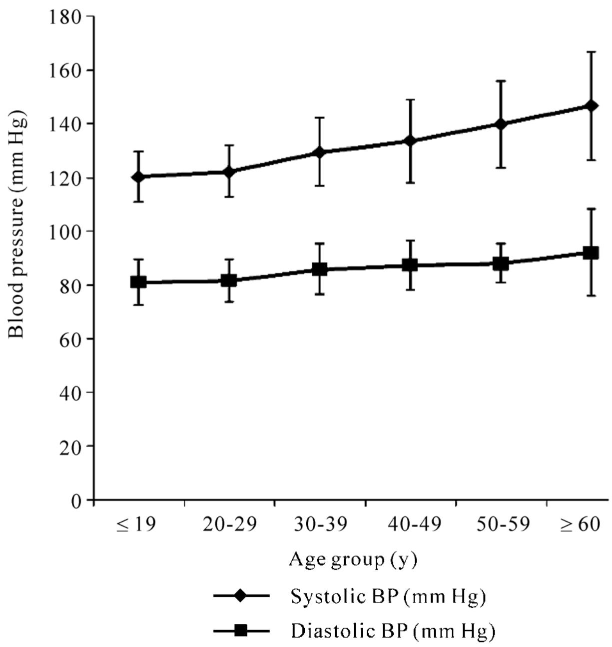

Figure 1. Systolic and diastolic BP by age group in Nsukka urban, Nigeria. Results are shown as mean and standard deviation.

4. DISCUSSION

This study presents the prevalence estimate of hypertension in Nsukka, an urban town in South-Eastern Nigeria.

Table 3. Blood pressure by age group and gender.

Age group (y)	n	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	n	Systolic BP (mm Hg)	Diastolic BP (mm Hg)
		Men (n = 364)			Women (n = 392)	
≤ 19	28	123 ± 10	81 ± 8	31	118 ± 8	82 ± 8
20-29	106	127 ± 9	85 ± 8	162	119 ± 9	80 ± 8
30-39	75	132 ± 12	88 ± 9	109	127 ± 13	85 ± 9
40-49	85	136 ± 15	88 ± 8	48	128 ± 16	86 ± 12
50-59	36	143 ± 15	89 ± 6	18	136 ± 17	88 ± 9
≥ 60	34	147 ± 17	94 ± 14	24	145 ± 25	90 ± 19

Results are presented mean ± standard deviation

Blood pressure showed a consistent increase with age in both men and women. The survey showed that only 30% of persons with raised blood pressure were detected, 21% were detected, while 9% were controlled.

Similar prevalence estimate have been documented in the literature. In South-Eastern Nigeria, prevalence of hypertension in a university community was as much as 21% [12]. Arterial hypertension was found in 25% of examined motor bike riders in Benin City, Nigeria [13]. It has been speculated that about 20%-25% Nigerian adults could be classed as hypertensive [14].

The consistent increase of blood pressure with age in both men and women is a known occurrence and has been reported elsewhere in Africa [15]. A recent study in Nigeria (Kogi State) showed that blood pressures increased with age and body mass index [16]. The age-related rise in systolic blood pressure is primarily responsible for an increase in both incidence and prevalence of hypertension with age [17]. It has been stated that the prevalence of hypertension increases with advancing age to the point where more than half of people aged 60 to 69 years old and approximately three-quarters of those aged 70 years and older are affected [1]. Though blood pressure increased with age, men had a significantly higher blood pressure than women in all the age groups.

The survey showed that only 30% of persons with raised blood pressure were detected, 21% were treated, while 9% were controlled. In general, our results showed a comparable level of detection, treatment and control of hypertension with those that have been reported in other surveys in Africa. For example in Ghana, it was reported that only 34% were aware that their blood pressure was high and 22.2% were taking antihypertensive medication, but only 6.2% had optimal blood pressure control [18]. Although a recent survey in Nigeria by Omuemu *et al*., reported a lower detection rate of 18.5% compared to 30% obtained in this survey, treatment and control rates in both surveys were comparable [19].

This study strengthens the fact that in sub-Saharan Africa, level of detection, treatment and control are still far less than results obtained in developed countries [20]. This poor level of detection, treatment and control has been attributed to scarce resources and inadequate healthcare provision [21,22]. The poor level of detection, treatment and control of hypertension is a cause of concern. As hypertension is an important cardiovascular risk factor, many undetected hypertensive patients have a high risk of suffering from cardiovascular disease consequences such as myocardial infarction and stroke. As was stated earlier, the burden of hypertension might be underestimated in Nigeria.

This study had some limitations. Stratified sampling technique would have been the best sampling method which will ensure the generalizability of the prevalence estimate. Also, since the blood pressure of the entire residents' (≥ 18 years) in the identified houses was measured, clustering effect could have resulted diluting the randomness of the sampling.

5. CONCLUSIONS

Prevalence of hypertension was comparable with other studies in Nigeria and Africa. Our results also showed a poor detection, treatment and control of hypertension than has been reported in other surveys in Africa. This underscores the need for comprehensive evaluation of prevalence of hypertension and other cardiovascular diseases in Nigeria. Information from this assessment could demonstrate the need to urgently address this emerging disease. It is also imperative to design cost-effective strategies which could be implemented to improve detection, adherence and control of hypertension in Nigeria.

6. ACKNOWLEDGEMENTS

The authors wish to acknowledge the following final year pharmacy students that took part in the field study: Ejike Onah, Ifeanyi Aduaka, and Theresa Money.

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Vol.2, No.7, 736-741 (2010)
doi:10.4236/health.2010.27112

Dynamic analysis of lymphocyte subsets of peripheral blood in patients with acute self-limited hepatitis B

Bo Liu, Jun Li*, Yaping Han, Yuan Liu, Lianhua Kong, Yang Cao, Zuhu Huang

Department of Infectious Diseases, the First Affiliated Hospital with Nanjing Medical University, Nanjing, China;
*Corresponding Author: dr-lijun@vip.sina.com

Received 19 March 2010; revised 12 April 2010; accepted 13 April 2010.

ABSTRACT

Purpose: To investigate dynamic changes and significance of lymphocyte subsets (T lymphocytes, B lymphocytes, NK cells and T cell subsets) of peripheral blood in patients with acute self-limited hepatitis B (AHB). Methods: Immune cells of peripheral blood were compared among 17 cases of self-limited acute hepatitis B patients, 36 patients with chronic hepatitis B (CHB) and 32 healthy controls by flow cytometry (FCM). $CD4^+/CD8^+$ was monitored dynamically, meanwhile relations between T lymphocyte subsets and ALT and clearance of HBV DNA were explored. Results: Dynamic changes of lymphocyte subsets were found in AHB, the level of $CD3^+$T cells was significantly higher compared to CHB group and healthy control group. Frequencies of $CD3^+CD4^+$ T cells in the third and fourth week and $CD4^+/CD8^+$ in the second week were higher compared to other groups. Frequency of NK cells was low and was significantly lower compared to other groups in the third week specially. It was showed that $CD4^+/CD8^+$ was low followed by high abnormal ALT during early stage by dynamic monitoring of $CD4^+/CD8^+$, and $CD4^+/CD8^+$ was increasing accompanied by normal ALT set by set, but $CD4^+/CD8^+$ had no significant relation to ALT and HBV DNA. Conclusion: Immune status of AHB, compared to CHB and healthy controls, was significantly different and dynamic changes of lymphocyte subsets may be related to progress of disease.

Keywords: Acute Hbv; Self-Limited; Lymphocyte Subsets; Facs

1. INTRODUCTION

Hepatitis B virus (HBV) is one of the most prevalent viral pathogens in humans, with almost a third of the world population having evidence of infection, and about 350 million chronically infected patients. Approximately 1 million people die annually from HBV-related disease, such as liver failure, cirrhosis and hepatocellular carcinoma [1]. After body was infected by Hepatitis B virus (HBV), complex immune responses could be caused, and cell-mediated immunity is an important factor to determine results of HBV infection [2]. Different subsets of lymphocyte have different responses to viral antigens and effects on clinical course and prognosis of development. Many precious researches focused on lymphocyte subsets in peripheral blood of chronic hepatitis B [3,4], which indicated that there was an imbalance in peripheral blood T-lymphocyte subsets and turbulence in cellular immunity. Researches about stable detection of lymphocyte subsets for adults with acute self-limited hepatitis B (referred to as “acute hepatitis B”) [5] showed that acute-phase CD4 responses were efficient and CD8 responses were multispecific irrespective of the outcome of infection. In this paper, by comparing differences of lymphocyte subsets among acute hepatitis B, chronic hepatitis B and normal controls, dynamic characteristics of lymphocyte subsets in acute hepatitis B were observed and the relationship between body’s immune response to HBV infection and disease prognosis was explored.

2. MATERIALS AND MEHTODS

2.1. Characteristics of Patients

Patients with acute hepatitis B, chronic hepatitis B were out-patient clinics and hospitalized patients of Department of Infectious Diseases, the First Affiliated Hospital with Nanjing Medical University from May 2007 to May 2008, diagnosis were in line with the revised diagnostic criteria of the Tenth National Conference on viral hepatitis and Liver Disease for viral hepatitis in 2000 [6] and the “prevention and treatment of chronic hepatitis B guide" [7], and exclusion of other hepatitis viruses and CMV, EBV, HIV co-infection. The information of patients was shown in **Table 1**.

2.2. Instruments and Reagents

IgG1-PE/IgG2a-FITC,CD3/CD4/CD8/CD45,CD3/CD4/CD28, CD3/CD8/CD28, CD3/CD19/CD56/CD45 monoclonal antibodies (fluorescent-labeled), as well as hemolytic agents were purchased from BD company(USA). Flow cytometry (BD FACS Calibur, USA). Automatic biochemical analyzer and its reagents were Beckman Company's products (USA). HBV DNA quantitative detection equipment were production of Roche's Light Cycler 1.0(USA).

2.3. Detection of Lymphocyte Subsets

Methods were referred Ya Pinghan [8] etc. and modified it slightly. 100 µl blood samples were collected into heparinized vacuum tubes and blended with mouse anti-human monoclonal antibody CD3/CD8/CD45/CD4, CD3/CD16 + 56/CD45/CD19 (fluorescent-labeled) 20 ul respectively, reacted at room temperature protected from light for 30 min, and was detected by FACS in 2 h. A "gate" of the leukocyte common antigen (CD45)-positive cells was set up according to forward and side scatter two-parameter point diagram and lymphocyte surface markers were analyzed by two-parameter. Statistical results were analyzed by use of CellQuest software.

2.4. Dynamic Detection of Lymphocyte Subsets

The frequencies of lymphocyte subsets were measured and dynamically analyzed for acute hepatitis B when admitted to hospital for the first, second, third and fourth week, compared to normal controls and chronic hepatitis B. Dynamical $CD4^+/CD8^+$ ratios were detected and dynamical relationships between $CD4^+/CD8^+$ ratios and alanine aminotransferase (ALT) were analyzed. In order to explore relationship between $CD4^+/CD8^+$ ratios and clearance of HBV DNA, $CD4^+/CD8^+$ ratios of acute hepatitis B with HBV DNA positive and negative patients were analyzed and compared.

2.5. Statistical Analysis

Measurement data were expressed using $\bar{X} \pm s$. By application of SPSS 11.0 statistical software, means of two groups were compared using *t* test or paired *t* test and multiple sets of means were compared by single-factor ANOVA analysis of variance. The correlation among ratios of lymphocyte subsets, alanine aminotransferase and HBV DNA level were analyzed by use of correlation analysis. $P < 0.05$ was referred as statistically significant.

Table 1. Some characteristics of different groups in patients.

Group	No	Male	Female	Average age
AHB	17	11	6	32.4 ± 4.8
CHB	36	20	16	37.5 ± 6.2
Normal	32	18	14	35.5 ± 3.9

A total of 17 cases of acute hepatitis B group, 11 males and 6 females, aged from 18 to 57 years old, and average (32.4 ± 4.8) years of age. Chronic hepatitis B group, 36 cases were recruited, 20 males and 16 females, aged from 23 to 75 years old, and mean (37.5 ± 6.2) years of age. Healthy controls, 32 cases without past and current HBV infection, as a normal control group, 18 male cases and14 female, aged from 22 to 53 years old, and mean (35.5 ± 3.9 years).

3. RESULTS

3.1. Changes of Lymphocyte Subsets for Acute Hepatitis B

For acute hepatitis B at admission and the dynamic changes trend of lymphocyte subsets for the first, second, third and fourth week (**Figure 1(a)**), and lymphocyte subsets of normal controls and chronic hepatitis B (**Figure 1(b)**).Peripheral blood $CD3^+$ T cells within 4 weeks of acute hepatitis B hospitalization were significantly higher, and the difference was statistically significant (**Figure 2**) compared to normal controls and CHB group. Frequencies of $CD4^+$ T cells from admission to 4th week showed a rising trend which were higher than normal controls and CHB group, and the differences of the third week and fourth week were statistically significant (**Figure 3**).For acute hepatitis B,$CD8^+$ T cells showed a upward trend first and then showed a downward trend, and the difference was statistically significant for the results of the second week (**Figure 4**).$CD56^+$ cells in 4 weeks were lower than both normal controls and CHB group, however, the third week compared with other two groups and the fourth week compared with CHB group respectively, the difference were statistically significant (**Figure 5**).

3.2. Level of ALT in Acute Hepatitis B and Changes in the Ratio of Lymphocyte Subsets

For acute hepatitis B, the trends of $CD4^+/CD8^+$ ratio means and ALT dynamic changes for hospitalized in the first week, second week, third week and fourth week were showed in **Figure 6**. For third week and fourth week, difference of $CD4^+/CD8^+$ ratio was statistically significant ($P < 0.05$) compared with chronic hepatitis B

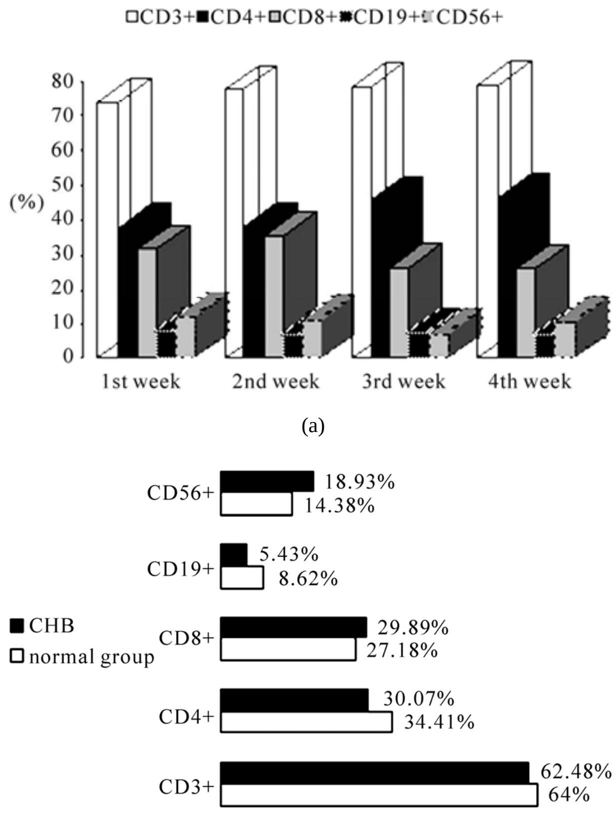


Figure 1. (a) Dynamic changes of acute hepatitis B lymphocyte subsets. Patients admitted to hospital and dynamic changes of lymphocyte subsets $CD3^{+}$,$CD4^{+}$,$CD8^{+}$,$CD19^{+}$ and $CD56^{+}$ for the first, second, third and fourth week. (b) Frequencies of lymphocyte subsets of normal controls and chronic hepatitis B.

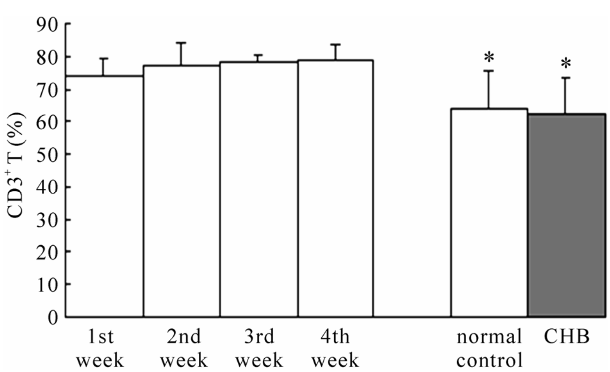
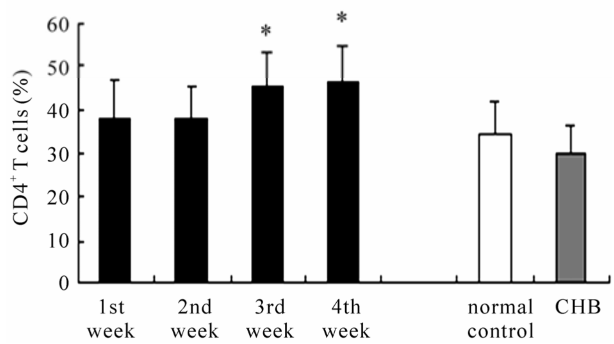


Figure 2. The results of dynamic changes of $CD3^{+}$ T cells in patients with AHB compared to normal controls and CHB. The mean ratios of different weeks for AHB were 73.97 ± 5.21%, 77.67 ± 6.64%, 78.54 ± 1.78% and 78.81 ± 5.01% respectively. And the ratios of normal controls and CHB were 64 ± 11.54% and 62.48 ± 11.33% respectively. Compared to the other groups, $CD3^{+}$T cells of AHB were higher and the differences were statistically significant. (*P < 0.05).



Finger 3. The results of dynamic changes of $CD4^{+}$ T cells in patients with AHB, compared to normal controls and CHB. For AHB, the mean ratios of $CD4^{+}$ T cells were 37.86 ± 9.15%, 37.93 ± 7.34%, 45.71 ± 7.42% and 46.37 ± 8.09% respectively. The mean ratios of $CD4^{+}$ T cells in normal control and CHB were 34.41 ± 7.53% and 30.07 ± 6.67% respectively. Compared to the other groups, $CD4^{+}$ T cells of AHB in third and fourth week were higher and the differences were statistically significant. ($^{*}P < 0.05$).

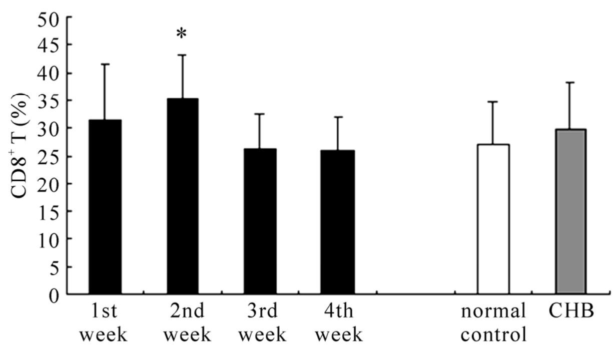


Figure 4. The results of $CD8^{+}$ T cells in patients with AHB compared to normal controls and CHB. The mean ratios of $CD8^{+}$ T cells in patients with AHB in four weeks were 31.58 ± 10.05%, 35.24 ± 7.79%, 26.45 ± 6.01% and 26.1 ± 5.95%, respectively.And the mean ratios of normal control and CHB were 27.18 ± 7.59% and 29.89 ± 8.36% respectively. The differences between 2nd week of AHB and the other groups were statistically significant. ($^{*}P < 0.05$).

(1.12% ± 0.28%).For chronic hepatitis B, difference was statistically significant (P < 0.05) compared with normal control (1.41% ± 0.60%). $CD4^{+}/CD8^{+}$ ratio and ALT changes were observed dynamically, and at early stage high levels of abnormal ALT followed lower $CD4^{+}/CD8^{+}$ ratio. As ALT normalization, the $CD4^{+}/CD8^{+}$ ratio showed a gradual increasing trend. The correlation between dynamic $CD4^{+}/CD8^{+}$ ratio and ALT was analyzed for different weeks, and r values were 0.700, –0.286, –0.179, 0.286, P values were 0.188, 0.535, 0.702, 0.535 (all > 0.05), respectively ,which suggested no significant correlation.

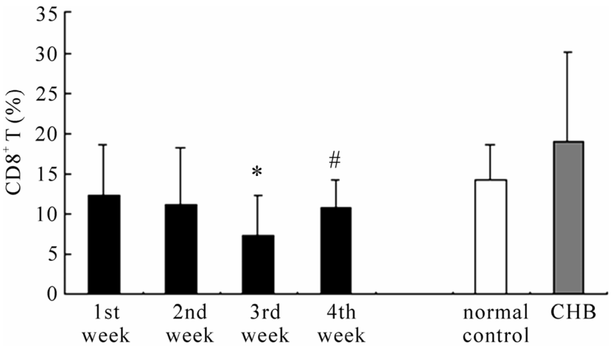


Figure 5. The results of $CD56^+$ cells in patients with AHB compared to normal controls and CHB. The mean ratios of $CD56^+$ cells in patients with AHB were 12.28 ± 6.32, 11.15 ± 7.12, 7.26 ± 5.04% and 10.89 ± 3.33 within four weeks, respectively. The mean ratios of normal control and CHB were 14.38 ± 4.23% and 18.93 ± 11.22% respectively. The difference between the 3rd of AHB and the other groups was statistically significant ($^*P < 0.05$) and the difference between the 4th of AHB and the CHB was statically significant. ($^{\#}P < 0.05$).

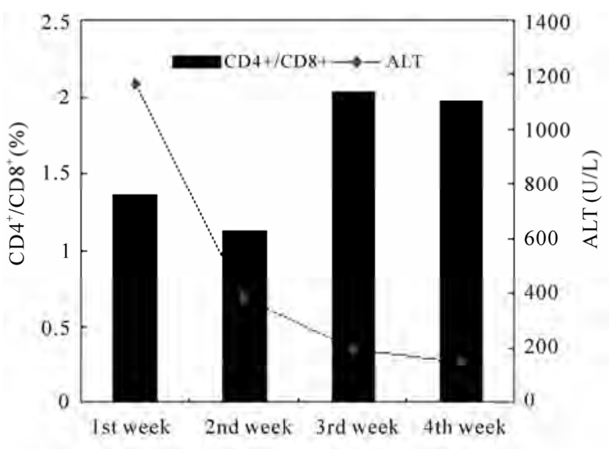


Figure 6. Trends of $CD4^+/CD8^+$ ratio means and ALT dynamic changes. It showed that ALT became gradual normalization followed $CD4^+/CD8^+$ gradually increased over time.

3.3. The Ratio of Lymphocyte Subsets and HBV DNA

HBV DNA of acute hepatitis B on admission were detected, of which 5 cases were negative (HBV DNA < 10^3 copies/ml) and 12 cases were positive (for the 10^3 copies/ml < HBV DNA < 10^7 copies/ml), and positive patients happened to be HBV DNA negative conversion within two weeks. The $CD4^+/CD8^+$ ratio of 12 patients with positive HBV DNA (mean 1.18% ± 0.93%) was lower than that of negative patients (mean 1.59% ± 0.47%), but no statistically significant difference was found between them (t = –0.701, P = 0.501).Twelve cases were positive in patients with $CD4^+/CD8^+$ ratio and the presence or absence of HBV DNA and no significant correlation (*r* = 0.433, P = 0.466). The $CD4^+/CD8^+$ of and HBV DNA of 12 cases of patients with positive HBV DNA were no significant correlation (r = 0.433, P = 0.466).

4. DISCUSSION

When adults are infected with HBV, the vast majority of people can clear virus and only a little infected persons continued to be chronic infection. Most studies have confirmed that the prognosis of HBV infection is closely related to functional status of the immune system which is an important factor to determine the prognosis. Acute hepatitis B triggers the body's immune response, particularly cell-mediated immunity. In our study, dynamic changes of peripheral blood lymphocyte subsets in acute hepatitis B and characteristics of peripheral blood lymphocyte subsets with normal controls and chronic hepatitis B were observed and analyzed, in order to initially explore the relationship between lymphocytes change and disease progression and prognosis.

$CD4^+$ T cells play a important role in viral clearance [9], and play a central role in controlling immune response. Subsets of $CD8^+$ T cells include cytotoxic T cells (CTL) and suppressive T cells (Ts), however, inactivated CTL are differentiated to special killing effective T cells dependent on identifying processing antigen and by IL-2 secreted by $CD4^+$ T cells. Past studies about lymphocyte subsets of peripheral blood such as Urbani S, D. Vassilopoulos and others [10,11] used more static detection, but we detected lymphocyte subsets dynamically. It showed that lymphocyte subsets showed a certain degree of dynamic change for acute hepatitis B: $CD3^+$ T cells increased significantly within four weeks of hospitalization and $CD4^+$ T cells showed increasing trend compared to normal controls and chronic hepatitis B. However, for chronic hepatitis B, $CD3^+$ T cells and $CD4^+$ T cells ratios were lower than normal controls. These figures showed that the weaker $CD4^+$ T cells immune response may be related to chronic infection. Differences of $CD3^+$ T and $CD4^+$ T cells ratios between acute and chronic hepatitis B suggested that when adults happened to be acute infection, T lymphocytes of peripheral blood, especially a higher proportion of $CD4^+$ T cells, may be related to a strong Th1 cell response. As disease progression, more obvious advantage of $CD4^+$ T cell ratio was essential to be ridding of virus. $CD8^+$ T cells ratio of acute hepatitis B had a peak, and was higher than other two groups, but the ratio has fallen and were lower than other two groups in third and fourth week. As for chronic hepatitis B, $CD8^+$ T cells slightly increased compared with normal controls. As we know, $CD8^+$ T cells have different subsets and most studies confirmed that for acute hepatitis B there was a strong polyclonal, multi-specific CTL response. In our study $CD8^+$ T cells had advantage of ratio during early stage, however, specific CTL proportion might explain the phenomenon, which injured body's own cells while clearing the virus. But for chronic hepatitis B, CTL response was not likely to dominate and Ts cell ratio mainly increased which inhibited cellular immune function, leading to sustained and persistent infection.

In part $CD4^+/CD8^+$ reflects state of immune system within a certain range [12]. The up-regulation of ratio indicates that immune response is strong and the reduction of ratio or even less than one indicates that low immune function. As for chronic hepatitis B, Yin Ying [13], You Jing [14] and so on confirmed existence of $CD4^+/CD8^+$ down. Our study showed that $CD4^+/CD8^+$ of acute hepatitis B in the first, third and fourth week were higher than that of normal controls and chronic hepatitis B which because activation and reproduction of specific CTL depending on $CD4^+$ T cells activated, and a higher proportion of $CD4^+$ T cells was behalf of inducing a strong anti-viral response. As $CD4^+$ T cells of acute hepatitis B increased significantly, compared to chronic hepatitis B, it indicated that arise of $CD4^+/CD8^+$ in acute hepatitis B showed advantage of positive regulation and a good prognosis disease.

For acute hepatitis B, ALT became gradual normalization followed $CD4^+/CD8^+$ gradually increased over time. Although a significant correlation between them was not found, a moderate cellular immune response happened during acute infection, which was conducive to completely remove virus, meanwhile which played a role in immune regulation function to restore tissue damage. HBV DNA was likely to have been largely cleared before liver damage for majority of acute hepatitis B, and for twelve cases of positive patients, HBV DNA continued to decline rapidly and serology turned to be negative. Virus was cleared mainly through hepatic cell disruption or non-dissolving mechanism [15]. For acute hepatitis B with positive and negative HBV DNA, $CD4^+/CD8^+$ of them had no statistical difference and HBV DNA level and $CD4^+/CD8^+$ also had no significant correlation. However as for chronic hepatitis B, You Jing [9] suggested that there was significant negative correlation between $CD4^+/CD8^+$ and HBV DNA level, which suggested that immune status of chronic hepatitis B was at a low level and viral replication was relatively stable, and immune response could not completely inhibit virus replication, leading to persistent infection. Nevertheless, for acute hepatitis B, whether positive or negative HBV DNA, immune response was at a relatively positive adjustment advantage, and a strong particularly cellular immunity, which could rapidly clear HBV DNA and play a strong and appropriate regulation function in later reaction, ultimately got to be clinical recovery.

NK cells are component of innate immune. They express molecular CD56 and play the role of first line of defense by leading to infectious cell disrupted non-specifically. Moretta L. *et al*. [16] confirmed that in early stage of acute hepatitis B, NK cells could play a role compared to chronic hepatitis B and normal controls. Our study showed that NK cells of acute hepatitis B had a high proportion in early stage, then dropped to a trough in the third week. NK cells of chronic hepatitis B was higher than normal controls. Chronic hepatitis B had a protracted course and persistent inflammation, leading to NK cells always maintained a relatively high proportion. For acute infection NK cells of peripheral blood showed dynamic changes, and it may be related to balance and accumulation of NK cells between blood circulation and liver tissue, so that it was more conducive for NK cells to produce IFN, and thus inhibited viral replication and cleared infected cells.

In summary, by dynamically analyzing lymphocyte subsets of peripheral blood in acute self-limited hepatitis B in a certain period of time, the relationship between clearing virus and immune system changes was explored. It showed that when acute hepatitis B occurred, there was a more comprehensive and effective immune response, which can be completely rid of virus and access to clinical recovery. However, for different individuals infected with HBV, how body activates a strong immune response in order to completely remove virus, as well as limitations of this study to a certain extent, additional studies are required further study in the future.

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Vol.2, No.7, 742-752 (2010)
doi:10.4236/health.2010.27113

Health

Barriers and facilitators to mexican-american participation in clinical trials: physician and patient focus group perspectives

Jesse Nodora[1], Tomas Nuño[2], Ken O'Day[3], Virginia Yrun[2], Francisco Garcia[2]*

[1]National Center of Excellence in Women's Health and the Arizona Cancer Center, University of Arizona, Tucson, USA
[2]National Center of Excellence in Women's Health and the Mel and Enid Zuckerman College of Public Health, University of Arizona, Tucson, USA; *Corresponding Author: fcisco@u.arizona.edu
[3]Xcenda, Palm Harbor, USA

Received 16 March 2010; revised 21 April 2010; accepted 22 April 2010.

ABSTRACT

Racial/ethnic minority populations are underrepresented in clinical trials and Hispanic participation rates are particularly low. This study assessed barriers and facilitators to clinical trials participation by Medicaid eligible Mexican-Americans and their serving physicians. Qualitative data from two focus groups conducted among Mexican-American Medicaid eligible patients and four physician focus groups were analyzed. Mexican-American patients have a basic understanding of clinical trials. While most are open to participating in clinical research, not speaking English, time, and transportation were identified as barriers. Physicians believe that desperation and financial need are the primary patient motivators for participation. Barriers to physician recruitment and referral include: lack of information about clinical trials, concern that study participation may not be in the patient's best interest, and lack of staffing and time to conduct trials. Ample opportunities exist to engage providers and patients in future efforts to increase Mexican-American patient recruitment into clinical trials.

Keywords: Clinical Trials Participation; Mexican-American; Hispanic; Medicaid Patients; Medicaid Physicians; Focus Groups; Qualitative Methods

1. INTRODUCTION

In a poll of clinical investigators conducted by Applied Clinical Trials, 56% of respondents identified participant recruitment as the most pressing issue in the conduct of clinical trials [1]. Difficulty in recruiting participants increases the time it takes to complete clinical trials, delays approval of new medications, and reduces incentives for drug development. Recruitment appears to be particularly challenging among racial/ethnic minority populations, and these groups tend to be seriously underrepresented in clinical trials [2]. Under representation of minority populations in clinical trials limits our understanding of the wide range of biological, social, and cultural factors that influence treatment response and reduces the generalizability of new treatments to minority groups; it thus may contribute to health disparities [3, 4]. Also, as a matter of equity, those suffering from disease should have access to new and promising treatments through clinical trials, regardless of their racial and ethnic identities.

As part of the National Institutes of Health Revitalization Act of 1993, the United States Congress mandated that women and minorities be included in clinical trials in a manner "sufficient to elicit information about individuals of both sexes/genders and diverse racial and ethnic groups" [5]. Despite this mandate, recent studies continue to show disproportionately low participation levels by minorities in clinical trials [6-9], and participation by Hispanics is especially low. For example, a study of US National Cancer Institute (NCI) sponsored clinical trials found that Hispanics were the most under represented racial/ethnic group [8]. Research into the factors responsible for under representation of minorities in clinical trials has largely focused on African-Americans, and comparatively little of this research has explored barriers among other ethnic racial/minority groups [6].

The process of recruiting patients to clinical trials involves both patients and the clinical investigators (or their representatives) who are responsible for presenting the trial for consideration by patients. Two recent reports on clinical trials recruitment among racial/ethnic minorities recommended more research to explore factors and mechanisms influencing patient-provider roles, especiallly those related to clinical trials communication [10,11]. Both reports suggested that clinical trials education is most likely to reach minority populations by providing tailored information to non-specialists primary health care providers in a community setting.

The term Hispanic includes individuals identified by the Office of Management and Budget Directive 15 as "A person of Mexican, Puerto Rican, Cuban, Central or South American, or other Spanish culture or origin, regardless of race" [12]. By 2050 it is estimated that one out of every four Americans will be of Hispanic ethnicity [13]. Persons of Mexican descent account for over 60% of all US Hispanics (the next closest group, Puerto Ricans, account for less than 10% of all US Hispanics) [14]. Also, Mexicans represent 32% of all US immigrants, the next closest group (Filipinos) are at 5% [15]. Although Hispanics share a common language, there are many cultural differences among the various sub-groups. In the present report, we focus on Mexican- Americans, the largest group of Hispanics in the US, and present the results of a series of focus groups designed to explore perceived barriers and facilitators to clinical trial participation among both Mexican-American patients and the physicians who care for them.

2. METHODS

We conducted focus groups with Mexican-American Medicaid eligible patients and their serving physicians to learn about barriers and facilitators to clinical trial participation. Structured open-ended interview-guided patient focus groups explored issues around patient barriers and facilitators to participating in clinical trials. Using the same technique, physician focus groups explored issues around patient barriers and facilitators and physician barriers and facilitators to recruitment and referral of patients to clinical trials. A total of six focus groups were conducted between May and August 2006. Two focus groups were conducted with patients, two with physicians that do not recruit or refer patients to clinical trials, and two with physicians that do recruit or refer patients to clinical trials. The study was reviewed and approved by the Institutional Review Board at the University of Arizona.

The study participants for the patient focus groups were Mexican-American Medicaid eligible patients. All were residents of Maricopa County in central Arizona. Patients were recruited by telephone from a comercially available list of Hispanic households. Patients were queried using a series of screening questions **(Table 1)** and were invited to participate if they: 1) made health care decisions for themselves or their family; 2) had interacted with the health care system within the past 18 months; 3) were between the ages of 30 and 65; and, 4) were Medicaid eligible (*i.e.*, had a household income of less than 100% of the Federal poverty level). The study participants for the physician focus groups were community primary care physicians who serve Medicaid patients. Physicians were recruited by telephone using a list of Medicaid eligible providers and were queried about whether they recruit or refer patients to clinical trials. Physicians that recruited or referred patients to clinical trials and those that did not recruit or refer patients were invited to participate in separately scheduled focus groups.

Trained interviewers conducted each focus group using a structured interview guide. The interview guide was developed and revised through an iterative process involving the above noted literature review of barriers and facilitators to patient participation in clinical trials. In addition, members of the research team conducted an expert review, and a sample of Mexican-American women visiting the Women's Health & Resource Center performed a community member review. Questions on the interview guide were open-ended and aimed to elicit participants' thoughts and feelings on various issues relating to clinical trials, recruitment, and participation. Patient interview guides also included questions about patients' awareness and attitudes about clinical trials, participation in clinical trials, barriers and facilitators to participation, and the role of culture and ethnicity. Physician interview guides included questions about patient barriers and facilitators, patient characteristics that affect patient willingness to participate, physician experiences in recruiting or referring patients, and physician barriers and facilitators to recruiting or referring patients.

The patient focus groups were conducted in Spanish and the physician focus groups were conducted in English. Focus groups ranged in size from 5 to 9 participants and each session lasted between 90 and 120 minutes. All focus groups took place in a focus group interviewing room with integrated audio recording equipment. All focus groups were audiotaped. Patient focus groups conducted in Spanish were subsequently translated and transcribed into English. Physician focus groups were transcribed verbatim in English.

Two members of the research team read and reviewed the transcribed focus group interviews for themes and codes in a two-part process. First, a concepttual model of factors affecting patient participation in clinical trials, based on a model developed for AHRQ, was revised and used to develop a set of themes and

Table 1. Patient and physician focus group questions.

Patient Questions (Spanish)

Healthcare system (El Sistema de Salud)

1. *Do you have a regular doctor that you see? In choosing your doctor what's important to you?* (¿Tiene un doctor que visita con regularidad? En elegir a su doctor, ¿que es importante para usted?)
2. *As a Hispanic, how important is it for you to have a doctor who is Hispanic or who understands your language and culture?* (¿Como Latino/a, que importancia tiene para usted tener un médico que es Latino o que entienda su idioma y cultura?)

Attitudes about Research and Clinical Trials (Actitudes Sobre las Investigaciones y los Ensayos Clínicos)

1. *What do you think about medical research?* (¿Qué piensan sobre las investigaciones médicas?)
2. *Who benefits from medical research?* (¿Quién beneficia de las investigaciones médicas?)

Participation in Research and Clinical Trials (Participación en las Investigaciones y los Ensayos Clínicos)

1. *Has anyone ever been asked to participate in a clinical trial or had a family member who was asked to participate in a clinical trial?* (¿Alguien de ustedes ha sido invitado a participar en un ensayo clínico o han invitado algún miembro de su familia a participar en un ensayo clínico?)
2. *Now, even for those of you who haven't ever been asked to participate, I'd like you to imagine a situation where you visit your doctor for some health problem and toward the end of your visit your doctor mentions that you might be eligible to participate in a clinical trial. How would you feel about participating?* (Ahora, para los que nunca han sido invitados a participar, imagínense una situación donde visitas a su médico y al final de su visita su médico menciona que quizás sea candidato para participar en un ensayo clínico. ¿Cómo se sentiría acerca de participar?)

Barriers and Facilitators (Barreras y Promotores)

Let's talk about some specific reasons that would influence you or someone in your family when making a decision about whether to participate in a clinical trial (Hablemos acerca de algunas de las razones que podrían influir su decisión, o la decisión de un familiar, para participar en un ensayo clínico)

1. *What are some of the reasons you might decide to participate?* (¿Cuáles son algunas de las razones para participar?)
2. *What are some of the reasons you might decide NOT to participate*? (¿Cuáles son algunas de las razones por cual NO participará?)

Culture and Ethnicity (Cultura y Origen Étnico)

1. *Do cultural and language issues influence your decision to participate in a clinical trial?* (¿La cultura y el idioma influyen su decisión para participar en un ensayo clínico?)
2. *Would being approached by a doctor who is Hispanic or who understands your language and culture influence your decision to participate?* (¿Influyera su decisión para participar siendo invitado por un médico Hispano o de habla Hispana que entienda su cultura?)
3. *Do you think physicians are more or less likely to ask Hispanic patients to participate in clinical trials?* (¿Piensa que es más, o menos, probable que los médicos piden a Hispanos que participen en ensayos clínicos?)

Physician Questions

Physicians Thoughts on Patients and Clinical Trials Participation

1. *How aware would you say your typical patient is of clinical trials? Do they know what they are for*?
2. *Do you think the existence of clinical trials is well communicated* (*to patients*) *in general*?
3. *When patients bring up the subject of clinical trials do they have any preconceptions*?
4. *What are your thoughts on your* (*Medicaid*) *patient's* [*knowledge, interest, and barriers/facilitators*] *related to clinical trials participation*?
5. *How does Hispanic culture influence clinical trials participation for Medicaid patients*?
6. *How can physicians increase clinical trials participation among their Hispanic Medicaid patients*?

Physician Facilitators and Barriers to Clinical Trials Participation

1. *Have you referred patients to clinical trials? Was this a positive or negative experience*?
2. *What is your interest in increasing your involvement in clinical research activities*?
3. *What would facilitate achievement of your desired research activity level* (*issues and solutions for physicians involved in research versus those not involved in research*)?

codes [6]. Transcripts were independently reviewed by two researchers, and passages were thematically classified as they related to the following patient and physician themes: awareness, attitudes, resources, and opportunities. The researchers then met to review these classifications and resolve disagreements. The result of this systematic review process was a set of passages from each transcript for each theme-code pair.

3. RESULTS

A summary of patient and provider focus group outcomes are shown in **Table 2** and more detailed results are presented in the following sections. Please note that the qualitative nature of focus group results does not easily allow for exact quantification of responses. As a general reference, when we state that "some," "many," or "most" participants provided a given response the approximate percentages are 40, 70 and 90 percent, respectively.

3.1. Patient Focus Groups

The two patient focus groups included a total of 13 Mexican-Americans who met eligibility criteria for Medicaid services. Most participants (77%) were women, and the mean age was 39 years.

3.1.1. Patient Awareness

Patients expressed a basic understanding that clinical trials involve research to determine whether medications and treatments are safe and effective. However, some patients believe that clinical trials involve practice by inexperienced physicians. None of the patients in our focus group sample had participated in a clinical trial, but some reported that friends or family members had participated.

3.1.2. Patient Attitudes

Most patients believe that clinical trials are good and help to advance science. The most commonly expressed reasons for not participating in clinical trials included: fear of adverse events, lack of trust in the physician, and being part of an experiment in which inexperienced physicians/health care providers are involved. A commonly expressed reason for considering participation was the development of more effective medications that would benefit the participant and others. Many patients expressed that they would like more information about clinical trials before making a decision about whether to participate. Most patients indicated that they would consult their family members to help them decide whether to participate. Also, most of the patients in the focus group indicated that they would be willing to participate and indicated that trust in their physician would be a significant factor in their decision.

3.1.3. Patient Resources

Time constraints and transportation pose barriers for some patients. For patients who lack access to health care or medications, obtaining access to care or medications through a clinical trial would be an incentive to participate. Many patients indicate that they would like better information to help them understand what clinical trials are about and they believe that television would be the best medium to inform and educate Mexican-American communities.

3.1.4. Patient Opportunities

Patients believe that being Spanish speaking (not speaking English) poses a significant barrier to participation and that translators are often inadequate or unavailable. Patients believe that as Mexican-Americans, they are less likely to be asked to participate in clinical trials, primarily due to the language barrier.

3.2. Physician Focus Groups

The four physician focus groups included a total of 26 doctors, of whom 6 (23%) were women and 4 (15%) were Hispanic. Their clinical practice specialties were 14 (54%) in family medicine, 6 (23%) in internal medicine, and 6 (23%) in other types of medical practice.

3.2.1. Physician Perception of Patient Awareness

Physicians think that patients have little awareness or understanding of clinical trials. Physicians have differing views regarding how a patient's level of education affects their understanding: some believe that Medicaid patients have more difficulty understanding what clinical trials are, while others think Medicaid patients are no different from other patients. Non-recruiting physicians indicate that patients sometimes bring up clinical trials seeking physician reassurance or approval before participating. Some non-recruiting physicians believe that patients tend to overestimate the likelihood of side effects from study medications.

3.2.2. Physician Perception of Patient Attitudes

Physicians believe that patients with severe disease and those who are in financial need are more inclined to participate in clinical trials. Physicians believe that their relationship with patients can be very influential upon patient attitudes toward participating in clinical trials. Some recruiting physicians indicate that patient fear of side effects is a barrier. Many non-recruiting physicians indicated that Mexican-American patients are private and may take some time to develop trust, but once trust is established they are very trusting of their physicians.

Table 2. Focus group patient and physician outcomes.

PATIENTS	
Facilitators	***Barriers***
Want to participate in clinical trials	Believe clinical trials are done by inexperienced physicians
Will ask physicians about clinical trials	Fear adverse effects and experimentation
With physician's trust will participate in clinical trials	Lack of trust in the physician
Believe clinical trials help advance science	Time constraints
Believe clinical trials help develop better drugs	Lack of transportation
Have basic understanding of clinical trials as research	Being Mexican-American (language & culture)
Use television to recruit Mexican-Americans	Speaking only Spanish
PHYSICIANS	
Facilitators	***Barriers***
Want more information on clinical trials	Get little information on clinical trials
When clinical trials provide physician assistance and needed resources	Do not know where to go for information on clinical trials
Community physicians can be effective in recruitment	Believe patients are likely to overestimate side effects
Electronic medical records	Believe patients know little about clinical trials
Clinical trial medications and procedures	Loss of patients to clinical trials
	Continued access to medication(s) after clinical trial ends
	Fear of being perceived as "on the take" by patients
	Lack of staffing and time
	Difficult to follow up some Mex. Am. Patients

Some recruiting physicians believe that Mexican-American patients are more difficult to recruit, but other recruiting physicians indicate that Mexican-American patients are no different from other patients in terms of their receptiveness to participation.

3.2.3. Physician Perception of Patient Resources

Economic hardship was thought to be an incentive for some patients to participate, but that it also makes clinical trials a low priority for patients who are experiencing economic hardship. Most physicians believed that transportation and distance are barriers for many patients, and that the impact of clinical trial participation on patient's employment may be potentially detrimental. Some recruiting physicians believe data collection burdens are a barrier to patient participation.

3.2.4. Physician Perception of Patient Opportunities

Some recruiting physicians stressed that there is considerable cultural variety among Mexican-American patients. Some physicians believe that their patient population's limited interaction with the health care system and the difficulty their offices encounter in contacting and following up with patients may limit opportunities for participation. Some thought that better advertising and getting interested community physicians involved in recruitment might also help improve patient opportunities.

3.2.5. Physician Awareness

Most physicians indicate that they get very little information about clinical trials and would like additional information about studies. Many physicians say that they do not know where to obtain information about clinical trials and are only aware of trials that they see ads for in the newspaper or hear about on the radio.

3.2.6. Physician Attitudes

Many physicians express concern about whether their patients would continue to have access to the study medication after the study is over. Some physicians are concerned about how they will be perceived by their patients if they recruit or refer them to clinical trials. A few of these physicians expressed ethical concerns about conflict of interest and that patients would perceive them as being "on the take," (*i.e.*, receiving inappropriate compensation for putting patients on a given study) particularly in the case of "skeptical minority" patients. Some physicians expressed general concern that participation in clinical trials may not be in their patient's best interest. Others voiced a specific concern that patients will be assigned a placebo intervention and that this might adversely impact their treatment plan. Some physicians are worried that referring or recruiting patients to clinical trials could potentially raise liability issues, or may lead to the eventual loss of the patient after the study is completed. Recruiting physicians believe that altruism is a limited patient motivation for participating. Physicians are more favorably disposed toward studies involving important new medications and less favorable toward studies involving "me too" drugs (*i.e.*, slight modification of an existing drug). Some physicians prefer to leave the subject of clinical trials participation for the patient to initiate and will only discuss the subject if the patient brings it up. Some non-recruiting physicians were quite favorably inclined toward clinical trials, while others are very skeptical. Some recruiting physicians said that they were motivated to recruit or refer patients in order to help them.

3.2.7. Physician Resources

Lack of staffing was identified by many physicians as a barrier to conducting recruitment and referral activities. Some of these physicians indicated that a research study coordinator would be necessary to conduct recruitment and referral activities in their office. Physician time constraints also limit the ability to recruit or refer patients. Some physicians were concerned that referring patients to clinical trials might result in a loss of patients for their practice, and a negative economic impact. Most physiccians indicated that assistance (e.g., administrative coordination, provider and patient materials) would be necessary for them to recruit or refer patients to clinical trials.

3.2.8. Physician Opportunities

Some recruiting physicians say that they do not receive much information about trials and have had to take the initiative to identify study opportunities for patient recruitment and referral. For many this represents an inordinate burden for routine referral of patients to clinical trials. Some recruiting physicians believe that electronic medical records have made it easier for them to participate in referring or recruiting patients by improving their ability to identify eligible patients and more easily transfer information.

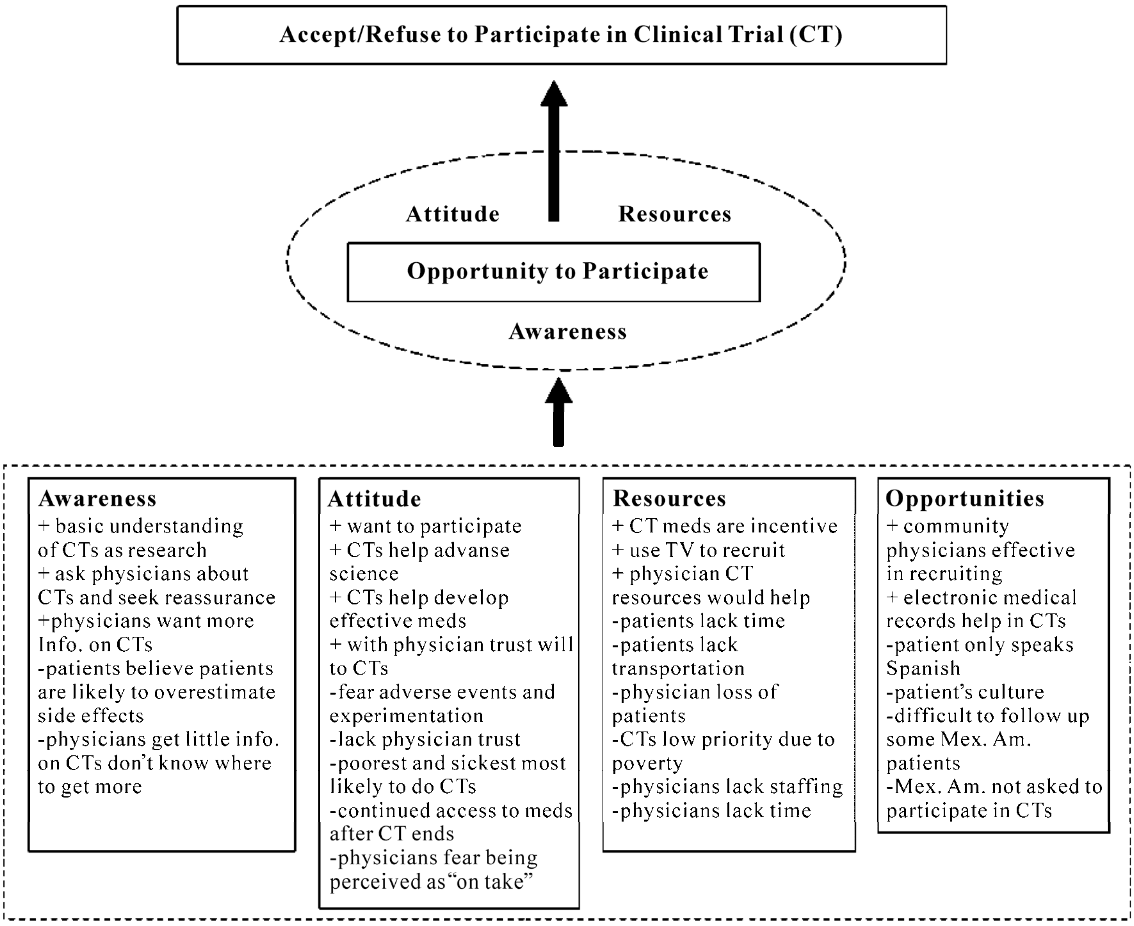
4. DISCUSSION

Without presenting the many research-related social (e.g., culture, racism) and demographic (e.g., race, ethnicity) complexities among such a large and heterogeneous group [4], it is clear that compared to non-Hispanic whites, US Hispanics are less educated, and more impoverished [16], and also face significant challenges in health care access, information, and knowledge [17]. This demographic and healthcare profile, coupled with the disproportionately low clinical trials participation rate, increases the urgency of understanding and addressing the barriers to Hispanic participation in clinical trials, especially among those of Mexican descent, which comprises the largest sub-group [14].

The results of our study on barriers and facilitators to Mexican-American clinical trials participation corroborate the findings of previous research; including many of those outlined in the recent Ford, *et al.* systematic review [2]. Our conceptual model, shown in **Figure 1**, is adapted from the Ford, *et al*. conceptual framework (**Figure 8**, p. 239) Our model integrates the key barriers and facilitators within each of four domains (awareness, attitude, resources, and opportunities) selected for this study. As depicted in **Figure 1**, we propose that the opportunity to participate in a clinical trial (for both patients and providers) must be present, and that the decision to accept or refuse is influenced by the facilitators and barriers in each of the four domains. The Ford, *et al*., model and our conceptual model share the Awareness and Opportunities domains. Based on our experience, and the iterative process described in the Methods, we chose to add the Attitude and Resources domains. These two domains provide an important link between awareness (*i.e.*, of clinical trial and what it offers) and action (accept/refuse to participate). In addition, the Resources domain captures many of the critical facilitators and barriers relevant to both patients and providers.

Our findings provide a concurrent perspective on clinical trials participation from both Medicaid eligible patients and the physicians who serve them. The impression from our study participants is generally favorable towards clinical trials research. Nevertheless, we found important facilitators and barriers among both patients and physicians **(**See **Table 2** and **Figure 1)**.

Both patients and clinicians are interested in clinical trials. Both express the practical barriers of time and need for assistance. The most complex, and often most difficult to overcome barriers for our participants revolve around not being fluent in English; where providers (and their staff) are not fluent in Spanish. While patients face fears of adverse effects and "experimentation," physicians face fears of being perceived as having a conflict of interest for referring patients to clinical trials and their patients not having access to medications once off study. Fortunately, many of the facilitators and barriers provide tangible opportunity for intervention.



Symbols: (+) indicates facilitator. (–) indicates barrier.

Description: Faced with the opportunity to participate in a clinical trial, consideration of facilitators (+) and barriers (–) within the four domains (awareness, attitude, resources, opportunities) will influence patient and provider acceptance or refusal to participate.

Figure 1. Focus group results applied to proposed conceptual model.

Most patients indicated that they were open to the idea of participating in clinical trials and believe that results of these help to advance science and medicine. This is consistent with recent findings from Markman *et al.* [18] and Wendler *et al.* [19] who found that both Hispanics, and African Americans, are as interested, or more interested, in learning about clinical trials as Caucasians. Efforts to educate and inform Mexican-Americans and other Hispanic patients about clinical trials should build upon this support and beliefs by clearly explaining the role of clinical trials in the development of new treatments that are safe and effective and emphasizing that the strict treatment protocols in clinical trials serve to ensure the provision of high quality care. Given that Mexican-American patients indicate that they would like additional information about clinical trials and believe television would be the best medium to reach them, development of appropriate television-based clinical trials messages may help to educate and recruit Hispanic patients.

There was near universal agreement among the patient focus group participants that language and culture pose significant barriers to participation in clinical trials for Mexican-Americans. There was also near universal agreement that fluency in Spanish is more important than the ethnicity of their physician. Most patients were, in fact, indifferent to the ethnicity of the physician. Some patients perceive Mexican-Americans as less likely to be asked to participate due to language barriers rather than discrimination. Such barriers may be reduced by providing physicians who serve Hispanic patients, as well as Spanish speaking study recruiters, with education and outreach on appropriate Spanish language information about clinical trials. Ramirez *et al.*, suggest not only outreach and education to address patient-provider language issues, but effective use of bilingual study teams [10]. Addressing language and culture may have the added benefit of facilitating family communication, understanding, and approval of clinical trials, which was identified as an important factor in patient decision making.

Of particular concern in our findings is that some of the participants believed that clinical trials involve experimentation, "practice" by inexperienced physicians, and frequent adverse events (*i.e.*, side effects). These findings are similar to those in a recent nationally representative 1000 person telephone survey, which found that African Americans and Hispanics are more likely than whites to associate clinical trials participation with more discomfort, pain, and side effects and to believe that they are better off with the standard treatment [9]. This is partially related to recent research on barriers to the recruitment of African Americans to clinical trials [20-24]. This literature presents the significant feelings of mistrust experienced by African Americans toward medical researchers due to a long documented history of research abuses in this population. While our study participants (both patients and providers) did not voice mistrust specific to historical reasons related to research abuse, physician trust was identified as an important factor for clinical trials participation. There are likely many complexities related to how a trusting relationship is achieved, not least among them, the ability to communicate (language), and cultural respect. This complexity creates many challenges to within and across group (African American-Hispanic) generalization, particularly in quantitative studies, and suggests the need to account for key associations (e.g., stratify by racial/ethnic origin, language, nativity, etc.) [25].

Limited patient resources—including time, transportation, and finances—appear to pose significant barriers to Mexican-American participation in clinical trials. This is not unexpected given that the patient focus group participants for this study were all under the Federal poverty level. While similar barriers may apply to low income non-Hispanic patients, any efforts to address the under representation of Mexican-Americans and other Hispanics in clinical trials will need to address these barriers due to the fact that these patients are disproportionately represented among low income and uninsured populations [26].

With concurrent patient and physician data lacking, results of the present study add an important dimension to the study of facilitators and barriers to clinical trials participation. Physician focus group participants, in the two non-referring/non-recruiting groups and the two referring/recruiting groups, were in general agreement on many topics and raised similar themes. The similarities appeared to be greater when physicians were discussing issues related to patient awareness, attitudes, resources, and opportunities. The larger differences between the non-referring/non-recruiting groups and the referring/recruiting groups tended to involve physician awareness, attitudes, resources, and opportunities.

Both physicians that do not recruit or refer and those that do, indicate that they get little information about clinical trials. This is particularly interesting in the case of those physicians who do recruit or refer patients. Generally, these physicians indicate that it is up to them to take the initiative to identify clinical trials. The fact that willing physicians are having to go out of their way to refer patients suggests that there are opportunities and a need for better forms of communication between researchers who are recruiting patients for clinical trials and community physicians. Physicians indicate that they would like more information about active clinical trials.

Physicians that do not refer/recruit, stated concerns about patients being placed on a placebo (rather than the study drug), the lack of patient access to the study drug after the study concludes, and that participation in a particular study may not be in a patient's best interest (e.g., of no direct health benefit). These physicians also express ethical concerns such as appearing to have a financial incentive or "bounty" for referring or recruiting patients. They were particularly sensitive about the appearance of exploiting minority patients. These kinds of concerns may help to explain why these physicians do not refer or recruit patients to clinical trials, especially commercially funded trials that may provide financial compensation for participant accrual. As suggested by Kim *et al.*, physicians, researchers and their institutions need to inform patients about financial conflicts in the same way they inform them about human subjects concerns [27].

Both groups of physicians were insistent that resource limitations adversely affect their ability to refer or recruit patients to clinical trials. Resources to enhance staffing (e.g., hiring a study coordinator) were thought to be essential to recruiting patients. Fear of losing patients was thought to be more of a concern for specialists who were referring patients to trials by other specialists, than referrals by primary care providers. Efforts to increase the proportion of community physicians who refer or recruit patients to clinical trials will need to provide physicians with additional resources and/or develop methods to enhance office and staff capabilities to refer or recruit patients. As expressed by participating physicians and Embi *et al.*, clinical practice tools such as electronic medical record systems may be useful in addressing these concerns [28,29].

Limitations of our work include small samples of patients and physicians that may not be representative of their respective populations. In our patient focus groups, for example, the majority of participants were women. A different group of participants may result in different issues being raised. Additionally, focus group research does not involve independent observations as participants influence one another within the context of the focus group. While this is an advantage of focus group research for exploring qualitative issues, it limits the generalizability of study findings. Also, analysis of focus group data involves a qualitative analysis of themes and, therefore, necessarily involves a subjective element despite attempts to be systematic and to eliminate bias (e.g., by using multiple coders). Strengths of our study pertain to the inclusion of both patient and physician provider focus groups in order to examine barriers and facilitators side-by-side. In addition, the Spanish language patient focus group protocols and questions were developed and implemented by trained and experienced bilingual and bicultural (Mexican-American) investigators and staff. This helps ensure linguistic and cultural accuracy of the study, as well as increased comfort and understanding, which in turn affects participant trust.

Recent forecasts predict that demand for clinical trials participants will outstrip supply [30]. Improving the recruitment of minority populations is critical to addressing the forecast shortage of clinical trials participants as well as ensuring the equitable distribution of the benefits and burdens of medical research. Currently only 11% of Arizona Medicaid (AHCCCS) primary care providers report that they recruit or refer patients to clinical trials (Appendix). With so few Medicaid serving physicians recruiting or referring patients to clinical trials, there are opportunities to engage more community physicians in recruitment and referral activities. Initiatives such as EDICT (Eliminating Disparities in Clinical Trials) conducted by the Baylor College of Medicine and the Intercultural Cancer Council (http://www.bcm.edu/edict/home.html), as well as those conducted by the Education Network to Advance Clinical Trials (ENACCT http://www.enacct.org/) provide ready-made resources for researchers, health care providers, an most importantly, patients and community leaders. As suggested by Robinson and Trochim [31], interventions that address community and researcher interests equally can help increase clinical trials participation among Hispanics and other underrepresented populations.

5. CONCLUSIONS

Although Mexican-American Medicaid eligible patients and the providers who serve them identify a variety of barriers to participation in clinical trials, facilitators for both groups validate the importance placed on clinical research and their willingness to participate. Interventions that provide clear, culturally relevant (*i.e.*, Mexican-American; primary providers serving Medicaid patients) clinical trials information, that addresses basic barriers such as time constraints, patient-provider communication and trust, are likely to increase accrual and retention.

6. ACKNOWLEDGEMENTS

This project was supported by a grant from the Arizona Biomedical Research Commission. The authors also wish to acknowledge the Behavioral Research Corporation which provided invaluable technical assistance with the focus groups, and the Arizona Health Care Cost Containment System leadership which provided assistance with the development of the study framework.

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APPENDIX

AHCCCS Provider Survey

In collaboration with our research team, the state Medicaid Agency (Arizona Health Care Cost Containment System-AHCCCS) agreed to include a question on its annual provider survey about primary care provider (PCP) participation in recruiting/referring patients to clinical trials. The item asked PCP respondents: *In the past three* (3) *years have you recruited or referred clients to clinical research trials*? The target population for the AHCCCS survey included a total of 7,656 providers, consisting of 2,633 PCPs, 2,999 specialists, 729 dentists, and 1,295 office managers. The survey was conducted by mail, web, and telephone from November through May 2006. A total sample of 1,764 surveys was completed, including 495 PCPs. The overall response rate was 52% and the response rate among PCPs was 51%. The table below summarizes the results of the PCP survey data by cross-tabulating responses of the clinical trials item with available data about provider characteristics (note: provider sex, race, and ethnicity data are not available from the survey). These data begin to provide a profile of the extent to which Medicaid physicians are involved in recruiting/referring patients to clinical trials. Based on this survey we know that only 11% of Medicaid providers currently refer patients to research studies. The only other statistically significant difference in participation rates relates to practice area; physicians in Maricopa and Pima (large metropolitan) counties are more likely to recruit or refer patients to clinical trials than in rural counties.

Table 1*. AHCCCS provider survey results.

	#	%
AHCCCS PCP Respondents	495	51%
Refer or Recruit Patients to Clinical Trials		
No	405	89%
Yes	52	11%
Refer/Recruit by Provider Type		
Family Practice	17	12%
General Practice	2	9%
Internal Medicine	14	11%
Pediatrician	19	12%
Refer/Recruit by Practice Area*		
Maricopa/Pima	45	14%
Other Counties	7	5%
Refer/Recruit by Health Plan**		
APIPA	42	12%
Care 1st	13	12%
Community Connection PHP	17	10%
Health Choice AZ	27	11%
Maricopa MC	10	13%
Mercy Care Plan	42	13%
Pima Health System	17	18%
University Family Care	8	21%

*Chi square = 6.5852, df = 1, $p \le .01$

**Since each physician can accept more than one health plan, this is not an unduplicated count

Vol.2, No.7, 753-758 (2010)
doi:10.4236/health.2010.27114

Health

Neuronavigation and epilepsy surgery

Martin B. Glaser[1]*, Konrad J. Werhahn[2], Peter Grunert[1], Clemens Sommer[3], Wibke Müller-Forell[4], Joachim Oertel[1]

[1]Department of Neurosurgery, University Hospital Medical Center, Johannes Gutenberg University, Mainz, Germany;
[*]Corresponding Author: glaserm@uni-mainz.de
[2]Department of Neurology, University Hospital Medical Center, Johannes Gutenberg University, Mainz, Germany
[3]Department of Neuropathology, University Hospital Medical Center, Johannes Gutenberg University, Mainz, Germany
[4]Institution of Neuroradiology, University Hospital Medical Center, Johannes Gutenberg University, Mainz, Germany

Received 16 February 2010; revised 11 March 2010; accepted 12 March 2010.

ABSTRACT

Resective epilepsy surgery is an elective therapy indicated in focal epilepsy patients who are resistant to pharmacotherapy. Every effort should be undertaken to perform the procedures as safe and less traumatic as possible. Neuronavigation could represent a suitable tool to reduce surgical morbidity and increase surgical radicality. Here, we present a series of 41 patients who were operated on for medically intractable epilepsy using neuronavigation. Overall, complication rate was 17% with a favourable seizure outcome of 88% (Engel's class I/II). Our data suggest that neuronavigation is a valuable surgical technique to accomplish a favourable outcome in epilepsy surgery.

Keywords: Neuronavigation; Epilepsy Surgery; Outcome

1. INTRODUCTION

Epilepsy is a frequent condition. Approximately 40 million people are affected worldwide and the prevalence of epilepsy has been estimated to be around 0.7% [1]. The mean annual incidence of first unprovoked seizures in population-based studies is 56.8 per 100 000 person-years, 23.5 per 100 000 person-years for single unprovoked seizures, and 33.3 per 100 000 person-years for epilepsy (recurrent unprovoked seizures). Partial seizures occur in 40-60%, two-thirds of which are temporal lobe epilepsies [2,3]. Clinically, focal epilepsy may first be suspected with a first witnessed report of a generalized tonic-clonic seizure but often seizures may be more subtle consisting of a transient short lasting loss of consciousness with or without oral or manual automatisms or focal tonic or clonic movements affecting parts of the body. Seizures may lead to developmental retardation, social impairment (e.g. limited choice of profession, ability to obtain a driving licence) and even sudden unexpected death in epilepsy [4]. In most cases, conservative treatment with antiepileptic drugs is successful in preventing clinical seizures, but up to 33% of patients will prove to be resistant to medical treatment [5].

Patients with focal epilepsy are generally surgical candidates, if medical treatment with at least two different anticonvulsive drugs in sufficient doses fails and disabling seizures persist. Bad prognostic factors for medical treatment in focal epilepsy are a structural lesion on Magnetic Resonance Imaging (MRI), particularly with dual pathology, post-stroke scars and vascular malformations having the best and cortical dysgenesis and hippocampal sclerosis the poorest outcome [3]. Optimal surgical results are obtained in patients with a circumscribed seizure onset (especially temporal/temporomesial) in video-EEG recordings, concordant focal pathology on MRI (e.g. hippocampal sclerosis) and concordant neuropsychological findings [6,7].

The need for a device enabling precise introduction of instruments into deep intracerebral structures was first addressed by Zernov *et al.* [8] 1890. He constructed a frame which was fixed on the skull by screws. The position of deep structures were measured from external anatomical landmarks. Clark developed 1908 a frame which served as a stable coordinate system for calculation of intracranial targets in relation to the frame [9,10]. In the second half of the last century these frames were refined. More and more indications were found along with the progress of the imaging modalities (x-ray, angiogram, computed tomography, MRI). Frame based stereotactic systems are still the most accurate navigational tools and very small targets like the subthalamic nucleus can be implanted with depth electrodes for the treatment of parkinsonism.

One major disadvantage of the stereotactic frame is the restricted surgical field as long as the arc is in place. At the end of the 1980s the "frameless" navigation was developed, with first clinical applications in neurosurgery at the beginning of the 1990s [11].

Nowadays, frameless neuronavigation is an accepted tool in contemporary microneurosurgery [12-15]. Its application contributes to make surgical approaches smaller and less invasive [16]. Consequently neuronavigation was integrated also in epilepsy surgery [17].

The neuronavigation is basically a miniature of a GPS (general positioning system). The neuronavigation systems are able to determine the position of the tip of a pointer in 3-D-space and to transfer the position into the appropriate CT or MRI data set in real time during the entire operation (in case of a microscope the focus corresponds to the tip of the pointer). From the technical point of view we can distinguish between armbased and armless navigation. The latter have the advantage not to restrict the operative field. Different armless systems were realized using sonic, infrared, magnetic waves or visible light (see **Figure 1**). The transfer of the pointer tip in the appropriate images makes a registration before application necessary. Per point registration and surface registration were developed for this purpose. The navigation devices have higher flexibility but less accuracy in comparison to the frame based systems. Regarding navigation accuracy we have clearly to distinguish between technical accuracy of the navigation system (how accurately the system determines the position in the 3-D-space), registration accuracy (how accurately is the data transfer from 3-D-space into the CT and MRI image space) and application accuracy depending of the intra-operative situation including brain shift [18].

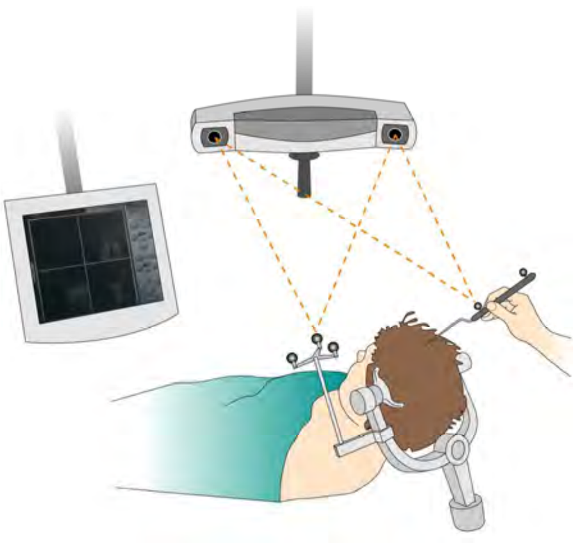

Figure 1. Drawing of an armless neuronavigation system setup.

For this study, we reviewed our surgical cases that were performed for pharmacoresistent focal epilepsy using a neuronavigation device.

2. MATERIALS AND METHODS

In our retrospective study, we gathered the clinical data of all patients who had navigation assisted surgery for medically intractable epilepsy. We evaluated the charts of 41 patients who were treated in our institution from 09.2003 to 08.2009 and reviewed the postoperative clinical follow up as well as neuro-imaging data for the degree of resection and complications.

Initially we used the Optical Tracking System (OTS®, Radionics, Burlington, Massachusetts, USA). In 31 cases, we navigated with the BrainLAB® System (BrainLAB, Heimstetten, Germany) and in a further 9 cases with the SonoWand® (Mison, Trondheim, Norway).

In frameless Neuronavigation, after general anaesthesia has been induced and immediately before surgery the patient's head is fixed in a three point fixation device and then referenced to the presurgical MRI (or other imaging modality such as computed tomography) by indicating to at least 4 defined landmarks so that the navigation system may locate the patient´s head in the three dimensional space. Hereafter the patient´s individual anatomy is shown on a monitor according to the region where a pointer is held on. The surgeon sees exactly where the targeted lesion is in relation to the skull surface to place the craniotomy on the ideal site. Moreover, he may check the position of his instrument any time during surgery.

For selective amygdala-hippocampectomies, we used a supraorbital craniotomy via a subfrontal approach [19]. Temporal pole resections with amygdala-hippocampectomies were approached via a small anterior temporal craniotomy (diameter approx. 2.5 cm). For extratemporal lesionectomies neuronavigation was also employed to gain direct access with craniotomies as small as possible. "Keyhole" approaches were applied when possible, especially in deeper seated lesions.

3. PATIENTS

This series includes 41 consecutive patients with pharmacoresistent focal epilepsy with a mean age of 36 years (15-70 years). There were 17 male and 24 female individuals. The mean duration of the epilepsy was 15.8 years. Most patients suffered from mesial temporal lobe epilepsy (n = 28, 17 left/11 right). All of them had been transferred from the department of neurology of the University Medical Center, Mainz, after video-EEG-monitoring for identification of the seizure onset region, correlation with the neuro-imaging and neuropsychological testing. Histological findings showed hippocampal sclerosis in 21 specimens. The remaining 7 had no specific changes (no abnormality, dysplasias, corpora amylacea).

The extra-temporomesial pathologies consisted of 4 gangliogliomas, 1 gangliocytoma, 2 astrocytomas, 1 oligoastrocytoma, 2 cavernomas, 1 gliosis after hemorrhage from an AVM, 1 dermoid and 1 meningeoma (6 left/7 right).

4. SURGICAL PROCEDURES

The surgery for the mesial temporal lobe epilepsy patients consisted of 2 selective amygdalahippocampectomies via a supraorbital subfrontal approach. The remaining 27 cases had an anterior temporal craniotomy for pole resection and amygdalahippocampectomy.

The extratemporal pathologies were approached by the shortest or least traumatic way concerning the patient´s neurological function. On the BrainLAB planning station, it is possible to determine the trajectory and import the information of the presurgical MRIs into the intraoperative surgical field.

In the operating room neuronavigation was installed after fixation of the patient's head in the Mayfield clamp. Accuracy was checked by correlation with anatomical landmarks after referencing the patients head with the preoperative 3-D-MRI data set either by laser or landmark registration (at least 4 points; mostly nasion, lateral orbital rims and upper helix attachments).

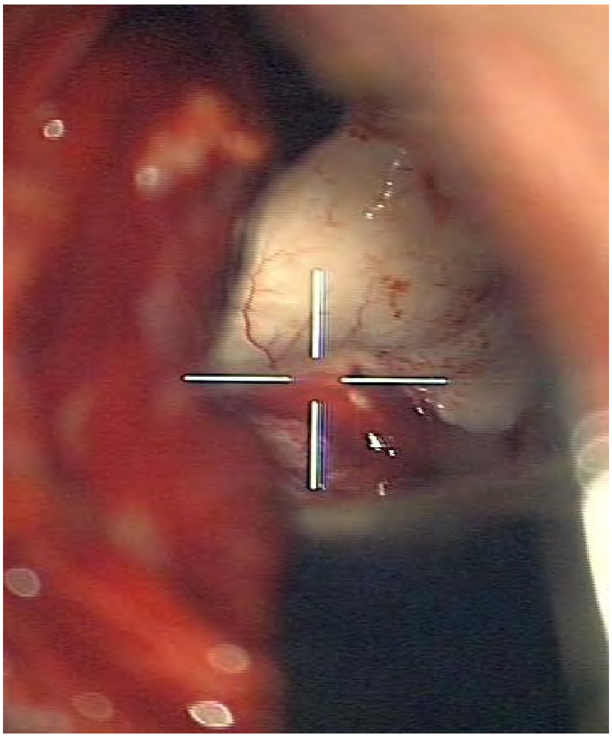

Figure 2. View of the hippocampus through the navigated microscope.

Neuronavigation was used to gain direct access to the pathological structures. This was achieved generally by use of a pointer. Additionally the microscope (Pentero or NC4, Carl Zeiss, Oberkochen, Germany) itself could be registered and navigated with the BrainLAB system. It was especially helpful in the amygdala-hippocampectomies in opening the temporal horn of the lateral ventricle to enable the dissection of the hippocampus. The viewing direction could be brought in the planned trajectory to reach the targeted structure. When the target is displayed in the ocular of the microscope, it is not necessary for the surgeon to place a pointer in the surgical field and look up to the monitor of the navigation system.

Finally the neuronavigation was then used to "define" the extent of resection of the hippocampus. It was intended to remove it at least to the dorsal edge of the cerebral peduncle.

5. DATA EVALUATION/FOLLOW UP

For all patients, site of surgery, duration between completed anaesthesiological preparation and skin incision as well as the time for the surgery itself, blood loss, ICU stay, hospital stay, neurological detoriation after surgery, degree of resection and seizure outcome were collected.

The follow up of the patients and the classification concerning Engel's epileptological outcome classes [20] were provided by the referring neurological department (KJW). Mean follow up time was 23 month.

6. RESULTS

Installation and usage of the neuronavigation systems was possible in all procedures. Average patient preparation (positioning, head fixation, referencing the neuronavigation, shaving, skin prepping, sterile draping) took 37 minutes. Mean duration of surgery was 209 minutes from skin incision to wound closure. The mean ICU stay scored 20.3 hours, the mean hospital stay 8.5 days. There was an average blood loss of 310 cc per complete procedure. Not a single blood product was administered.

There was no mortality in this series. The following complications were noted: One patient had a space occupying frontal epidural haematoma on his routine postoperative cranial computed tomogram which was clinically asymptomatic but evacuated for its size. Two patients showed a slight hemiparesis caused by small thalamic ischemias. They regained full strength but still have a deficit in fine motor skills. A further two patients had incomplete oculomotor palsies which resolved without sequelae. One patient developed a severe generalized vasospasm 10 days after subtotal frontal lobectomy. He has no focal neurological deficit but a relevant lack of motivation.

One rhinoliquorrhoea occurred after a supraorbital approach via the opened frontal sinus. The liquorrhoea ceased after temporary lumbar drainage.

Postsurgical imaging showed complete removal of the extratemporal pathologies in 9 of the 13 cases. The degree of hippocampal resection was noted in relation to the brain stem: a relatively short resection of the hippocampus only to the middle of the cerebral peduncle was performed in 5 cases, to the dorsal margin of the cerebral peduncle in 20 cases and in a further 3 cases beyond.

The neuronavigation was sufficiently exact in all cases at the beginning of the procedure. Accuracy was as reliable with laser patient registration as with registration via anatomical landmarks. The calculated mean deviation was 1.7 mm. It was possible to reach all lesions/ structures that were aimed for. It was extremely helpful in localization of the temporal horn in amygdala-hippocampectomies. Neuronavigation overestimated the degree of resection of the hippocampus, possibly due to brain shift after CSF loss-especially after opening of the lateral ventricle.

Postoperative seizure outcome was favourable after amygdala-hippocampectomy with 21 patients Engel's class I and 6 patients Engel's class II. One patient was seizure free for 3.5 years and developed pharmacoresistent temporal lobe epilepsy again so that re-resection is being considered.

In the patients group with the extratemporal resections, 11 patients became seizure free (Engel's class I). Two patients did not profit at all and have still the same seizure frequency in comparison to the presurgical state (partial tumor resections).

In total, antiepileptic drugs were discontinued in 8 patients and reduced in 5. The majority of 29 patients is still under medication, similar to presurgical status.

7. DISCUSSION

For decades, atraumatic surgery for medically refractory epilepsy has been the objective in order to improve patients functions and at the same time effectively reduce seizures. Neuronavigation contributes to that aim by minimizing the craniotomies and reach the target in the planned trajectory [13].

On the other hand, there are only few publications concerning neuronavigation and resective epilepsy surgery [17,21-25].

Previous reports on neuronavigation in epilepsy surgery were published without discussing its advantages and pitfalls or without giving any clinical data [26-28].

Wurm *et al.* [24] published the largest series of 140 patients who underwent surgery for medically intractable epilepsy. After the procedure for miscellaneous pathologies surgeons answered a questionnaire to assess the impact of the neuronavigation. They concluded that the application of the navigation system was effectively and safe in terms that the targets, even small in size, could be located precisely and electrodes could be placed accurately as well. Moreover the approach could be individually tailored.

In a previous series of Oertel *et al.* [22] neuronavigation seemed to be helpful in avoidance of complications (8% vs. 22%). In 93% the surgeon rated the application of the neuronavigation as "helpful".

A comparison of the complications in various studies is compiled in **Table 2**, seizure outcome in **Table 3**.

In our series complication rate and seizure outcome are comparable to larger series [29]. The application was safe. There were no complications with direct referral to the use of the navigation system. The time for preparation of the navigation was acceptable: in our evaluation the total time from anaesthesia induction to skin incision was 37 minutes. In comparison to that the installation of the neuronavigation equipment alone took additional 26 minutes in another study [30]. Surgery itself was not prolonged.

Table 1. Usefulness of neuronavigation.

Presurgical planning/strategy	Helpful for studying patients individual anatomy
Determination of craniotomy site	Helpful, especially over convexity
Locating lesions	Helpful, especially in subcortical pathologies
Amygdala-hippocampectomies	Extreme helpful in access the temporal horn
Resection control	Variable (brain shift), often overestimation, consider alternatives (e.g. ultrasound)
Delicate site of surgery	Helpful, shows eloquent structures as well

Table 2. Epilepsy surgery and complications (perm. = permanent; trans. = transient).

Complications	Acar *et al.*	Oertel *et al.*	Cho *et al.*	Glaser *et al.*	Sindou *et al.* without Navigation
CSF fistula			2 trans.	1 trans.	
Visual field defects	4 perm.	Not investigated	1	4 perm.	Not investigated
CN palsy		1 trans.		2 trans.	
Motor deficit	1 perm.	1 trans.		2 trans.	2 perm.
Aphasia	1 trans.	1 perm.		1 trans.	
Postop. haematoma			1	1	3
Infection					3
	n = 39	n = 38	n = 46	n = 41	n = 100

Table 3. Seizure-outcome after epilepsy surgery.

Engel's class	Acar *et al.*	Oertel *et al.*	Cho *et al.*	Glaser *et al.*	Sindou *et al.* without Navigation
I	37 (95%)	20 (53%)	28 (61%)	32 (78%)	85 (85%)
II	2 (5%)		10 (22%)	4 (10%)	9 (9%)
III			6 (13%)	2 (5%)	2 (2%)
IV			2 (4%)	3 (7%)	4 (4%)
	n = 39	n = 38	n = 46	n = 41	n = 100

8. CONCLUSIONS

Based on these results and our experience in the use of neuronavigation, we conclude that the application of a navigation system in epilepsy cases is safe and helpful in finding the targeted structure and in minimizing trauma to the patient by smaller craniotomies.

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Vol.2, No.7, 759-768 (2010)
doi:10.4236/health.2010.27115

Health

Costing of Malaria treatment in a rural district hospital

Insa Koné, Paul Marschall*, Steffen Flessa

University of Greifswald, Faculty of Health Care Management, Greifswald, Germany;
*Corresponding Author: Paul.Marschall@uni-greifswald.de

Received 7 January 2010; revised 18 February 2010; accepted 20 February 2010.

ABSTRACT

Objective: It is the aim of this paper to estimate the provider's cost of treating paediatric cases of Malaria in a rural African hospital. Furthermore, we intend to give some insights into the possibilities of improving the efficiency of treating children with this disease in order to support policy makers in the resource allocation process. Methods: The cost analysis was done in the district hospital of Nouna, Burkina Faso. Based on a comprehensive cost-of-illness information system, the cost of treating paediatric Malaria in the district hospital in the year 2005 were estimated using a combination of top-down and activity-based costing. It divides the entire treatment process into a set of activities along the clinical pathway and allocates monetary values for the resource consumption to each activity. Results: The average actual provider's cost were 6.74 US$ for a paediatric outpatient with Malaria, 61.08 US$ for a paediatric Malaria inpatient with anaemia and, respectively 74.29 US$ for a case of paediatric Malaria with neurological affection. 54% of the cost was due to laboratory work. This high unit cost was mainly due to a severe underutilisation of the hospital capacity. The current cost recovery rate per case was between 18% and 43%. It would be between 32% and 73% if the occupancy increased to 80%. Conclusion: The paper demonstrates that detailed costing is possible in a district hospital in rural Africa. The unit cost seems to be extra-ordinary high and the share of laboratory cost is tremendous. However, this is mainly due to a very strong underutilisation of the existing capacities. This fact calls for intensive efforts of the management of the institution to attract more patients by improving the quality of services and in particular the satisfaction of the patients.

Keywords: Burkina Faso; Cost Analysis; Clinical Pathway; Cost Recovery; Paediatric Malaria

1. INTRODUCTION

Malaria is one of the most common diseases and a major obstacle for economic and human development in sub-Saharan Africa (SSA) [1,2]. In particular children in this region suffer from high morbidity and mortality caused by Malaria [3]. For instance, in Burkina Faso in the year 2005 54.94% of the hospitalisations of children under the age of five were due to Malaria and the disease was with 57.29% the leading cause of death of under-five-years-olds [4]. In the health district of Nouna in the North-West of Burkina Faso Hammer *et al.* [5] analysed the causes of mortality for children and found that Malaria was the most frequent diagnosis (42%) in this district for the years analysed (1999 to 2003).

Although the magnitude of human suffering due to Malaria is well known and a wide range of research about Malaria prevention and effective treatment is ongoing, there is very little knowledge about the cost of treating Malaria in hospitals. Compared with the magnitude of studies on the epidemiology of Malaria in SSA, the number of studies focusing on the cost of this disease in hospitals is extremely low. Provider's cost per paediatric inpatient were estimated at 86 US$ (user fees 43 US$) in a tertiary hospital in Senegal for the year 1996/97 [6] and at 57 to 105 US$ in a district hospital and 33 to 44 US$ a sub-district hospital in Kenya for the year 1993/94 [7]. Recently, Ayieko *et al.* [8] estimated provider's cost of treating paediatric Malaria in district hospitals in Kenya between 47 and 75 US$ for the year 2005 without distinction between mild and severe cases. In the Ivory Coast total user fees for a hospitalised paediatric Malaria inpatient were estimated and resulted in 15 to 40 US$ per child [9].

The majority of studies build on a snap-shot cost analysis in the hospitals and not a routine costing system. There is an urgent need to determine the cost-of-illness of Malaria and in particular of the cost of Malaria patients in hospitals in this region based on a professional routine cost-of-illness information system.

This paper intends to contribute to the process of filling this gap. The motivation for this research is based on the conviction that proper resource allocation and an efficient roll-back of Malaria will only be possible if we know the cost of this disease so that managers can make informed decisions. The scope of the paper is limited to paediatric Malaria in Nouna district hospital, Burkina Faso. This example was chosen because a comprehensive cost-of-illness information system was established in this district in 2003 and Malaria has been an international research subject in this region for many years. The cost-information system covers direct and indirect cost as well as cost of first-line facilities and the district hospital. The methodology of the information system and the basic costing results were described elsewhere [10, 11]. The restraint to paediatric cases seems appropriate as the majority of grown-up Malaria patients already have semi-immunity and are generally treated as outpatients in rural health centres.

For this paper we used the existing cost information system and extracted the cost of treating paediatric Malaria patients in the district hospital to calculate provider's cost per patient. Consequently, the second section of this paper describes the costing methodology based on the actual patient's pathway in Nouna district hospital. Section three presents the results and section four discusses the consequences of these results for the hospital management. The paper closes with a few conclusions.

2. METHODOLOGY

The methodology applied for calculating the treatment cost of paediatric Malaria patients in the district hospital of Nouna is a combination of top-down and activity-based costing.

The top-down costing methodology was first developed for commercial commodities where up to 90% of cost is variable (e.g. cost of materials) and the rest (overheads) can be allocated proportionally to the costing units. This costing methodology has also been applied to hospitals [12-14]. A common approach is to divide the total cost of the institution by the number of patient days in order to determine the cost per patient day. The cost per patient is computed by multiplying the length of stay by the average cost per patient day.

However, up to 80% of total cost of hospitals is fixed and does not vary with the number of patient days. Therefore, allocating fixed cost to the diseases and patients according to the length of stay induces a severe error. In particular, the top-down approach does not allow any judgement on the impact of increased or decreased work load of an institution as fixed cost are proportionalised. Also, the methodology does not allow to distinguish patients with different diagnosis at the same ward and often gives only a rough picture of the real cost of a patient with a specific diagnosis.

Therefore, advanced costing methodologies have been established for the service industry and in particular for hospitals. They divide the entire treatment process into several activities or sub-processes and calculate the total cost of a particular patient or diagnosis by adding up the cost along the clinical pathway [15]. This activity-based (or bottom-up) costing has become a standard in the developed world [16-18], but it is hardly applied in hospitals of developing countries as it is quite detailed and requires a degree of precision of documentation and recording that is frequently not existing in these countries [19,20].

In a nut-shell, the top-down costing approach is faster than but not as precise as the activity-based costing. On the other hand activity-based costing requires very detailed cost information which is hardly available in developed countries and even less in developing countries. Consequently, a combination of top-down and bottom-up costing was chosen to calculate costs at a paediatric ward for the specific diagnosis Malaria with the available cost data. This mixed approach has been applied to similar problems before, e.g. [21].

The first step was the analysis of the existing costing data. The provider cost information system has been established in the hospital since 2003. As for standard step-down cost analysis cost centres were defined, with the difference, that any department directly in contact with the patient (e.g. ambulance, laboratory) was defined as final cost centre. The only overhead cost centres were laundry service and technical service. Cost for overhead cost centres were allocated to the other cost centres (e.g. administration, wards, laboratory etc.) according to the workload and added to the cost per service unit of a particular cost centre (e.g. cost per laboratory test). Intermediate cost centres were not defined as all other cost centres provided services which could be directly linked to the patient. Within each of the final cost centres a variety of products were defined, e.g. at the laboratory 44 different tests. Cost for each of them were calculated by the ingredient's approach, where the final price is the product of the quantity of inputs used and their value per unit [22,23]. This means that wherever possible cost were directly allocated to a specific laboratory test (staff costs per minute, equipment, consumption material) only overhead cost (electricity, building etc.) were divided by the total number of laboratory tests as in a top-down analysis.

The second step was the design of a standard pathway of paediatric Malaria cases in the hospital. For this purpose, we analysed 40 files of respective in- and outpatients of the year 2006 with Malaria as diagnosis. In addition, we interviewed the personnel in charge (physician, nursing officer, head of laboratory, drug seller, head of technical service, chauffeur, head of laundry) to triangulate the findings [24,25]. Finally, direct observation provided information of workload and material consumption of procedures (ward round, laboratory tests etc.). The fact that we chose patient files from 2006 was due to a lack of reliable files for 2005. However medical staff reassured us that there had not been major changes in treatment during this period. Based on our findings a standard pathway was developed in accordance with the guidelines for Malaria treatment for Burkina Faso [4] and of the World Health Organisation [26]. The comparison with these guidelines seemed appropriate as we suspected under-provision due to patients' financial straits. Possibly, treatment is sometimes abandoned before its time as the patient runs out of money. It was however our intention to estimate cost for a complete treatment.

The third step was to calculate the total cost per patient by summing up the cost along the clinical pathway.

In addition, total user fees were calculated. As patients have to pay fees for particular services, the total fees were calculated by adding up all user fees along the standard pathway.

The cost of treatment in rural health centres, the cost of pharmacies, private transportation and indirect cost (such as cost of food, accompanying family members, lost labour time etc.) were not considered. Drug cost within the hospital were only considered when directly associated with Malaria and its complications, e.g. anaemia. Antibiotics, vitamins and others were left aside.

Within the cost centres variable and fixed cost were distinguished (**Table 1**) and the cost behaviour of all cost categories was analysed. Variable cost rise proportionally with service units (e.g. any further patient) while fixed cost do not change [14,27]. Drugs, for instance, are consumed proportionally to the number of patients and can be allocated directly to a particular patient or diagnosis. On the other hand, the cost for equipment are fixed and will not increase if more patients are hospitalised. Consequently, the average fixed cost per patient will decrease with a growing occupancy as the cost are distributed among more patients (fixed cost degression).

Electricity was judged to be in parts fixed and in parts variable. On one hand, for example, the lightning needed for a 6-bed-bedroom does not depend on the number of patients occupying it (it remains the same, whether occupied by one person or by six persons). On the other hand, usage of medical devices accounts for a proportional rise in electricity with any further usage.

Table 1. Cost behaviour of different cost categories.

	Fixed cost	Variable cost
Building depreciation	100%	
Consumables		100%
Electricity	50%	50%
Equipment/vehicles depreciation	100%	
Fuel		100%
Pharmaceuticals		100%
Salaries and wages	100%	
Technical services	100%	

3. RESULTS

Table 2 gives an overview of total cost in cost centres related to paediatric Malaria for the year 2005 [28].

Based on this data from the cost information system we estimated unit cost for the services along the standard pathway including ambulance transport from a rural health centre to the hospital, laboratory tests, a bed day at the paediatric ward, medical and nursing care per day, drugs and the administrative procedure per patient from the provider's point of view as described above.

Figure 1 demonstrates the standard pathway of a paediatric Malaria patient in Nouna hospital. Although, the clinical perception of every patient is different, the series of sub-processes in the inpatient department is quite similar for almost all patients. The patient enters the paediatric ward, either because he is referred to the hospital by a rural health centre or because the relatives (usually parents) themselves decide to bring the child directly to the hospital. If necessary the hospitals ambulance is sent to fetch the child at the rural health centre. The first resources within the hospital are consumed by the consultation at the paediatric ward including anamnesis and physical examination. At the same time, registration involves the consumption of administrative time.

Depending on the general state and the Malaria symptoms the child is either admitted or treated as an outpatient. The standard treatment of an outpatient with milder Malaria is either Sulfadoxine/Pyrimethamine or Amodiaquine and an antipyretic agent in oral form. A thick blood film/blood smear is done at the laboratory to affirm the suspected diagnosis.

If the child is hospitalised an inpatient file is opened by the nurse or the physician, and medical and nursing

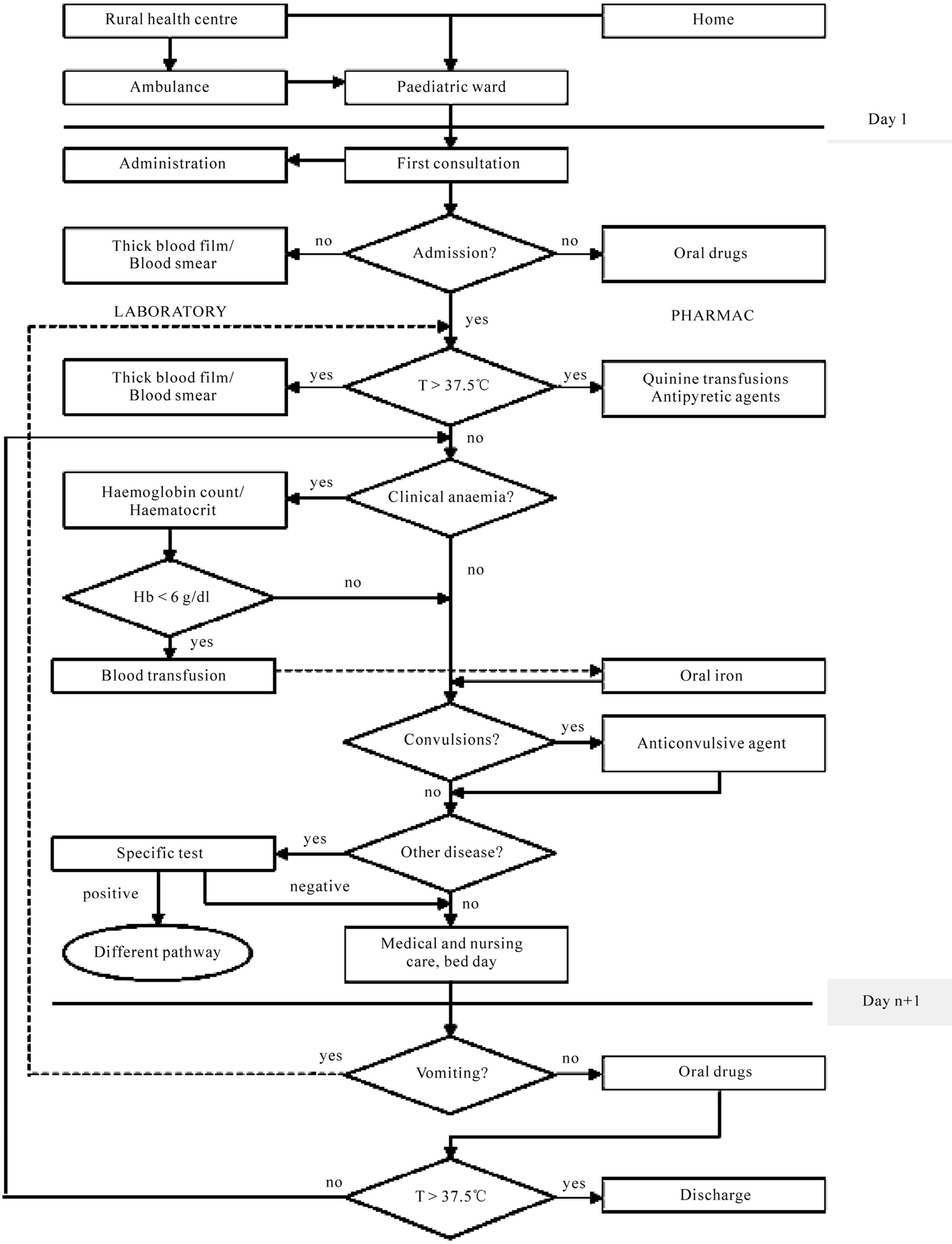

Figure 1. Standard pathway of paediatric Malaria patients at Nouna district hospital.

Table 2. Total cost of cost centres involved of paediatric Malaria treatment [US$] in Nouna hospital in the year 2005.

Department	**Building depreciation**	**Equipment/vehicles depreciation**	**Salaries & wages**	**Consumables**	**Technical services**	**Fuel**	**Pharmaceuticals**	**Total**
Administration	449	0	6.404	1.121	19.720	4.358	0	32.050
Electricity	41	0	0	0	19.582	0	0	19.623
Laboratory	2.166	22.950	10.582	4.245	0	0	0	39.943
Laundry	0	0	450	0	0	0	0	450
Paediatrics	971	473	12.910	0	0	0	0	14.354
Pharmacy	196	6	2.239	83	0	0	50.655	53.179
Technical services	62	136	3.370	0	2.489	0	0	6.057
Transport	0	0	0	0	0	0	0	0
Total	3.885	23.565	35.955	5.449	41.791	4.358	50.655	165.658

care starts. Directly connected with the examination is the preparation of blood-samples for the laboratory. Laboratory tests are asked according to clinical findings, e.g. paleness leads to a haemoglobin count/haematocrit. At the same time, parents or other accompanying relatives are instructed to buy drugs from the hospital pharmacy according to the clinical symptoms. Hospitalised Malaria cases receive intravenous anti-Malaria treatment with Quinine. The main symptoms of severe Malaria at the district hospital are anaemia (Hb < 6 g/dl) and convulsions. Clinic diagnosis of anaemia is confirmed by a haemoglobin count/haematocrit going along with a blood grouping. Blood transfusions are done accordingly, if the haemoglobin level is below 6 g/dl. Donors are mostly family members, thus cost occur only for blood collection, infectious screening and blood grouping. As long term treatment the children receive oral iron as substitute. Convulsions are treated with Diazepam. As Malaria symptoms are non-specific and might also be due to other infectious diseases, e.g. a gastro-enteritis or intestinal parasites, further laboratory tests can be required, e.g. blood count or white blood cell count or/and lumbar puncture. The examination of the patient's general state is repeated daily in form of the ward round and if necessary further laboratory tests are asked or/and drugs prescribed accordingly. The discharge depends on the general state of the patient. To give two important features, the child should by then be able to swallow and take an oral anti-Malaria agent and the body temperature should have dropped below 37.5°C. In the year 2006, the average length of stay of a paediatric inpatient with severe Malaria was 3.5 days (standard deviation 1.4) for cases with anaemia and 7.25 days (standard deviation 2.2) for cases with neurological affection. An average first consultation took 15 minutes.

Along the standard pathways costs were summed up assuming either an case of mild Malaria treated as an outpatient (**Table 3)** or the case of severe Malaria with either anaemia or neurological affection (**Table 4**). Cost resulted in 6.74 US$ for a paediatric outpatient with mild Malaria and 61.08 US$ for severe Malaria with anaemia and 74.29 US$ for Malaria with neurological affection.

The extra-ordinary high cost of the laboratory (54% on average) call for more analysis. A closer look reveals that the depreciation of equipment accounts for 64% of the total cost and that almost 88% of the laboratory cost are fixed cost (**Figure 2**). Considering the manpower it can be estimated that the number of tests performed could be increased by a factor of at least four without bringing the laboratory staff to their capacity limit. The combination of high fixed cost and low utilisation rate explains why the actual cost are quite high.

User fees are shown in **Figure 4**. An outpatient pays

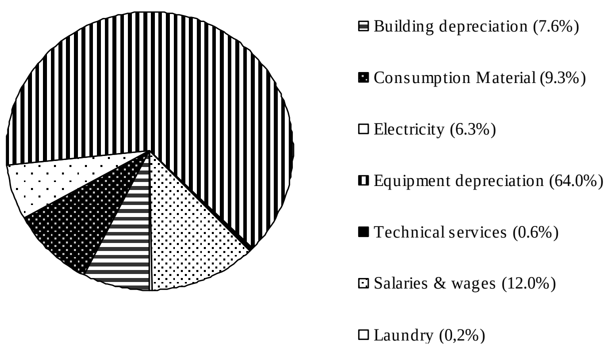

Figure 2. Distribution of laboratory cost in Nouna hospital in the year 2005.

Table 3. Cost for standard outpatient paediatric Malaria case in Nouna hospital in the year 2005.

	Unit cost (US$)	Quantity per patient	Cost per patient (US$)
Consultation			**1.94**
Staff	0.60	1	0.60
Overhead cost	1.34	1	1.34
Laboratory			**3.36**
Thick blood film/Blood smear	3.36	1	3.36
Drugs			**0.68**
Administration	0.76	1	**0.76**
Total			**6.74**

Table 4. Cost for standard paediatric inpatient case with severe Malaria in Nouna hospital in the year 2005.

		Anaemia		**Neurological affection**	
	Unit cost (US$)	Quantity per patient	Cost per patient (US$)	Quantity per patient	Cost per patient (US$)
Ambulance	**7.49**	**0.00**	**0.00**	**1.00**	**7.49**
Paediatric ward			**13.83**		**28.64**
Medical care/day	0.48	3.5	1.68	7.25	3.48
Nursing care/day	1.19	3.5	4.17	7.25	8.63
Bed/day	2.28	3.5	7.98	7.25	16.53
Laboratory			**41.14**		**31.97**
Thick blood film/Blood smear	3.36	2	6.72	2	6.72
Haemoglobine count/Haematocrit	8.00	1	8.00	0	0.00
Blood grouping	4.26	1	4.26	0	0.00
Blood count	4.90	1	4.90	1	4.90
Stool smear	2.94	1	2.94	1	0.00
Blood transfusion	14.32	1	14.32	0	0.00
Cerebrospinal fluid	18.63	0	0.00	1	20.35
Pharmacy			**5.36**		**5.43**
Administration			**0.76**		**0.76**
Total			**61.08**		**74.29**

2.88 US$ and inpatients pay 16.29 US$ in case of severe Malaria with anaemia and 13.37 US$ in case of neurological affection. This results in a cost-recovery rate of 43% for an outpatient and 27% and 18% for the inpatients. The low cost-recovery for Malaria with neurological affection is due to the fact that the examination of cerebrospinal fluid (CSF) is done within the scope of a research project about meningitis and therefore free of charge for the patient.

4. DISCUSSION

This paper investigates to calculate the cost of treating paediatric Malaria based on a standard pathway and activity-based costing as an example for an appropriate method to estimate the COI in a resource-poor setting.

Cost per case in Nouna district hospital are comparable to what was found in similar settings in Senegal and Kenya [6-8]. However, this should not mislead the management to assume that the situation in Nouna hospital is sound. Instead, there seems to be substantial wastage of resources, namely expensive devices and labour time of staff due to underutilisation.

High expenditures at the laboratory in Nouna are particularly due to high fixed cost particularly for equipment. The hospital is, in general, in a rather poor condition. However, the laboratory's building is brand new and holds up-to-date equipment not yet written off. Consequently, this department has comparably high cost. In addition, the department is strongly underutilised.

What was shown exemplarily for the laboratory is also valuable for other cost centres. If the hospital utilisation rate increased from currently 20% to 80% (NB: an occupancy between 80 and 85% is internationally seen as a professional standards [18]), the cost per inpatient would decrease to 34.59 US$ (paediatric Malaria with aenaemia) and 42.35 US$ (paediatric Malaria with neurological affection), whereas the cost for an outpatient visit would decrease to 3.95 US$. Cost per bed day at the paediatric ward (including building, equipment and overhead cost) would decline by 64% and laboratory cost would decline by 54% per patient. **Figure 3** shows the cost for the standard treatment per patient against the occupancy rate.

Assuming that user fees per patient would not change in case of higher occupancy rates, cost-recovery would increase to 73% for outpatients and 47% (paediatric Malaria with anaemia) resp. 32% (paediatric Malaria with neurological affection) for inpatients.

The costing method applied might furthermore mask higher fixed cost for medical and nursing staff. As we allocated staff cost according to the time spent for a certain patient, we do not account the additional free time caused by the lack of patients. If we divided total

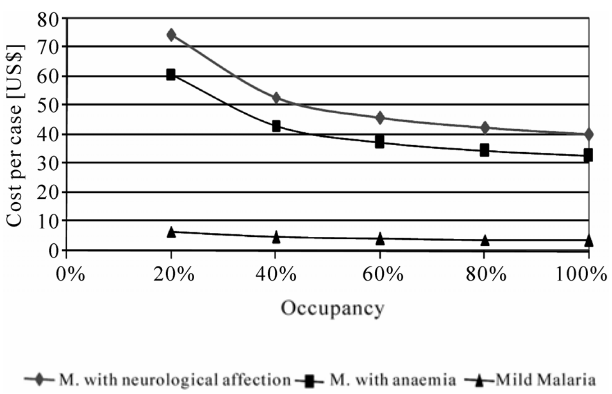

Figure 3. Cost for standard treatment against occupancy rate in Nouna hospital in the year 2005.

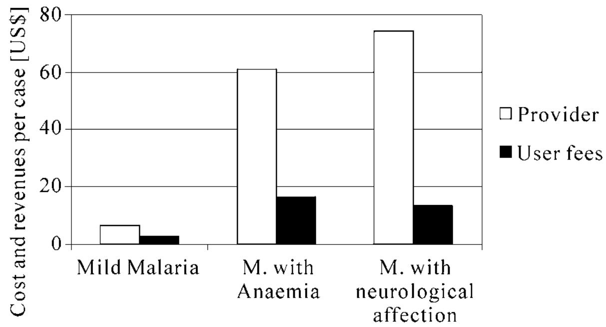

Figure 4. Provider's cost and user fees in Nouna hospital in the year 2005.

staff cost at the paediatric ward or at the laboratory by the number of patients or tests, cost per unit would be even higher.

The low occupancy rates and general underutilisation of health care in Nouna health district [29-31] and Burkina Faso as a whole [32] lead to high average cost per patient especially for staff [11,33]. To improve efficiency utilisation should be enhanced.

Possible reasons for low utilisation might be existing patients' dissatisfaction with the quality of health services [30,34-36] leading to a preference towards traditional healers or self-treatment. Baltussen *et al.* [37] found that the quality of care perceived at Nouna hospital was even worse than that of rural health centres.

Further reasons for low utilisation rates might be the distance to the health care facilities, as well as the influence of financial barriers [31,38]. Although longer distances to rural health centres can be avoided even in rural areas by appropriate planning [39] there does not seem to be an easy solution for the distance to the hospital.

User fees, on the other hand, were introduced according to the Bamako Initiative to make health care sustainable. Furthermore the correlation between better quality and increasing utilisation rates was shown by Litvack and Bodart [40] and Mariko [41] and was stronger than between abolition of user fees and utilisations rates. Thus management efforts have to focus on the quality of health care services in Nouna as elsewhere in SSA.

Our findings underline the results of other authors that a standard pathway is a useful tool not only for costing, but also to ameliorate the quality of care in cooperation with the health personnel [24,42] by establishing a standardised sequence of interventions along the patient's hospital stay. Criteria should be defined under which investigations are required within predefined time-frames. Any performed investigation has a consequence for the ongoing treatment and if at a certain point the treatment does not show the expected effects alternatives are foreseen. Furthermore, prefabricated patient files based on the clinical pathway could be developed, which simplify documentation. Also, total cost can be estimated already at the moment of admission and provide planning reliability to the health care provider as well as to the patient or his accompanying relatives. In Guinea-Bissau, for instance, it was shown, that a standardised protocol for the management of paediatric Malaria can lead to a decrease in mortality and average hospital stay, when going along with monitoring and a financial incentive for the staff [43].

5. CONCLUSIONS

Activity-based costing is an appropriate method to calculate the COI even in resource-poor settings and thus can be an important starting point to investigate inefficiencies.

Higher occupancy rates are the crucial point for a more proper resource allocation and a more efficient Malaria treatment in Nouna district hospital. Further development of the clinical pathway might be a positive incentive to improve quality of care and thereby render the hospital more attractive for the population to accentuate demand for modern healthcare. Increasing the utilisation of the district hospital would also help to make the hospital more sustainable as cost-recovery rates would considerably augment. Nevertheless, it is hardly possible that the hospital in its current configuration will break even.

A major share of total provider's cost is due to fixed cost. Increasing utilisation helps to lower average cost per case, ceteris paribus. Furthermore, unnecessary cost should also be avoided. For example before investing in new equipment for example at the laboratory, it should be verified if further devices are adequate for a district hospital in a developing country.

6. SHORTCOMINGS

We are aware that the suggested oral Malaria treatment changed lately to ACT (Artemisinin-based combination therapy) to control the growing resistance against anti-Malaria agents [26]. This treatment option was not available at Nouna district hospital in the years 2005 and 2006. The introduction of ACT might slightly increase provider cost for drugs, but does probably not change total treatment costs substantially as drug cost stand only for a small part of total provider's cost.

It was not possible to evaluate the level of patients' unofficial payments. It is likely they do pay for better or faster treatment, especially since considerable "hidden cost" were discovered in other low-income countries [44].

On the other hand, exemption mechanisms for indigents and pupils were also not considered when calculating total user fees and cost-recovery rate.

7. ACKNOWLEDGEMENTS

We would like to thank the German Research Society (DFG) for funding this study as project of SFB 544. We would also like to express our gratitude towards Ali Sié, Germain Savadogo, Dimitri Poda and Athanase Pacéré for supporting data collection for the cost information data base.

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Vol.2, No.7, 769-772 (2010)
doi:10.4236/health.2010.27116

Health

Alternations in salivary glucose during ramadan fasting

Reyhaneh Sariri[1]*, Abdolali Varasteh[1], Ali Erfani[2]

[1]Department of Biochemistry, The University of Guilan, Rasht, Iran; *Corresponding Author: sariri@guilan.ac.ir
[2]Department of Internal Medicine, Iran University of Medical Sciences, Tehran, Iran

Received. 21 October 2009; revised 7 December 2009; accepted 9 December 2009.

ABSTRACT

During the holly month of Ramadan, Muslims fast every day from dawn to sunset. Although the effect of Ramadan fasting on general health has been widely studied, the impact of fasting on oral health and possible changes in salivary biochemicals, such as glucose, has not received much attention. The aim of our study was to evaluate the influence of fasting on the level of glucose in the saliva of healthy individuals. Salivary glucose was measured using an enzymatic method based on oxidation of glucose by glucoseoxidase followed by determination of resulting H_2O_2 in the presence of peroxidase. A reduction in mean concentration of glucose was observed in the saliva of all fasting subjects as compared to the control group. It was concluded that reduction in salivary glucose is mostly due to reduced food intake and may be beneficial to dental health.

Keywords: Saliva; Glucose Level; Diabetes; Fasting

1. INTRODUCTION

Several of the world's great religions recommend a period of fasting or abstinence from certain foods. Of these, the Islamic fast during the Muslim month of Ramadan is strictly observed every year. During the month of Ramadan, Muslims fast every day from dawn to sunset. Muslims observing the fast are required to abstain not only from eating and drinking, but also from consuming oral medications and intravenous nutritional fluids.

It has been found that, in the healthy subject, Ramadan fasting does not appreciably affect one's health. However, it may induce some complications in patients with important metabolic disorders such as diabetes. The effect of experimental short-term fasting on carbohydrate metabolism has been extensively studied [1,2]. Mean normal blood glucose levels in humans are about 90 mg/dl, equivalent to 5 mM (mmol/l). It has been found that a slight decrease in serum glucose (60-70 mg/dl, 3.3-3.9 mM) occurs in normal adults a few hours after fasting has begun. However, the reduction in serum glucose ceases due to increased gluconeogenesis in the liver [3].

Saliva is the first biological fluid to encounter any change in eating habits as well as any environmental or physical changes. Saliva influences oral health both through its non-specific physico-chemical properties, as well as through more specific effects [4]. Saliva is well known for its highly protective functions against deterious agents such as microorganisms, toxines and various oxidants [5,6]. The antioxidant capacity and reducing power of saliva may diminish to a high degree due to various factors [7]. It has been shown that *in vitro* exposure to cigarette smoke could significantly decrease some enzymatic activities, both in plasma and in saliva [8,9].

The research reports in this area are few and almost limited to the changes of glucose concentrations in plasma. This study reports alternations in glucose content of saliva during one month fasting in Ramadan 1428 (Sept. 22nd-Oct 12th 2007).

2. MATERIALS AND METHODS

2.1. Materials

The level of glucose in saliva was measured using an enzymatic method based on oxidation of glucose by glucoseoxidase followed by determination of resulting H_2O_2 in the presence of peroxidase. An enzymatic assay glucose kit was purchased from Pars Azmoon™. The kit consisted of a standard glucose and a colour reagent. The concentration of standard glucose was 100 mg/dl and the colour reagent was a mixture of the following chemicals: 250 mmol/l phosphate buffer pH 7.5, 5.0 mmol/l phenol, 0.5 mmol/l 4-aminoantipyrine 10 ku/l glucose oxidase and 1 ku/l peroxidase.

2.2. Volenteers

30 healthy male students (mean age 24.21 years, s_x = 2.34), who intended to fast the whole Ramadan. 30 samples for control and comparison were also collected from the same students who donated their saliva in the month before Ramadan when they performed normal eating pattern. A precise consent was obtained from each individual and a dentist examined their mouth and teeth before sample collection.

2.3. Collection of Saliva Samples

The subjects were examined by a dentist for the absence of infection and other symptoms of oral/and or dental disorders. A few volunteers with severe infections were excluded from the research. After gaggling their mouth with about 5.0 ml of distilled water for about 2 minutes, timed un-stimulated whole saliva samples (3 ml) were collected in clean, dry in sterile pre-weighted tubes. The duration of saliva sampling was altered among individuals depending on their flow rate (2.0-5.0 minutes). The flow rate was calculated by measuring the time required to collect one ml of saliva in minutes. During the holly month of Ramadan, saliva samples were donated mid-day (after about 6 hours fasting) and three samples collected from each volunteer during one month of Ramadan (days 1-9, 10-19 and 21-29). Three control samples from each volunteer were also collected at the same time of the day during a non-fasting period, i.e. one week after Ramadan. All of the saliva samples were immediately centrifuged at 800 × g for 10 min at 4°C to remove squamous cells and cell debris. The resulting supernatant was stored at –18°C until used for determination glucose content. They were analyzed within 48-72 hours of collection. Each assay was repeated three times and the data obtained were expressed as mean ± SD of the three determinations. To test the effect of freezing on the glucose content of saliva, random measurements on fresh samples were also performed. No significant difference was observed between thawed and fresh samples. Therefore, only the frozen samples were used for continuing studies.

2.4. Glucose Assay

Glucose concentration was determined in supernatant of saliva samples collected from volunteers. The level of glucose in saliva was measured using an enzymatic method based on oxidation of glucose by glucose oxidase (GOD) followed by determination of resulting H_2O_2 in the presence of peroxidase (POD).

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$2\ H_2O_2 + \text{4-Aminoantopyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimini} + 4\ H_2O$$

1000 ml of reagent was mixed with 30 ml of each saliva sample. The mixture was incubated in a 37°C water bath and the intensity of resulting colour was measured spectrophotometrically at 546 nm against a blank of containing 1000 ml reagent and 30 ml distilled water.

Glucose concentration (C_G) in whole saliva was calculated using the Beer-Lambert's equation and absorbances of standards solution (A_S), each sample (A_T) and concentration of standard (C_S).

$$C_G\ (\text{mg/ml}) = A_T/A_S \times C_S\ (100\ \text{mg}/100\ \text{ml})$$

2.5. Statistics

Each assay was repeated triplicate and the results were presented as mean ± SD values. Statistical difference between groups was compared by un-paired t-test, p values less than 0.5 were retained as significant.

3. RESULTS

The saliva flow rate ranged from 0.08 to 1.40 ml/min at rest and showed about 10% decrease in response to fasting. Concentration of glucose was calculated in mg/100 ml using the absorbance and standard concentration. **Figure 1** compares content of glucose in saliva of some selected volenteers during fasting and non-fasting period. The data presented in this figure are a random selection from saliva samples of 30 volenteers. It should be emphasized that the results of other samples were similar to the randomly selected ones. The mean values together with p values are shown in **Table 1**.

4. DISCUSSION

The effect of experimental short-term and Ramadan fasting on carbohydrate metabolism has been extensively

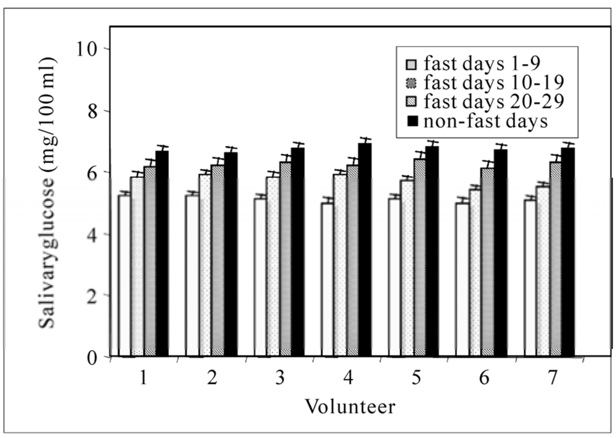

Figure 1. Comparison of glucose level in saliva samples of non-fast (control) with fasting group during different days of Ramada.

Table 1. Mean ± SD salivary glucose (mg/100 ml) of subjects during fasting nad non-fasting period.

Sample collected during	Salivary glucose concentration (mg/100 ml)	P values*
First 10 days of Ramadan	54.5 ± 0.74	0.5
Second 10 days of Ramadan	58.8 ± 1.25	0.6
Third 10 days of Ramadan	63.6 ± 9.43	0.5
First week after Ramadan	68.5 ± 1.22	NS

Values presented as Mean ± SD.

* P values were compared by t-test; NS – not significant

studied [10-13]. It has been found that a slight decrease in serum glucose to 60 mg/dl to 70 mg/dl occurs in normal adults a few hours after fasting has begun. However, the reduction in serum glucose ceases due to increased gluconeogenesis in the liver. In this study, some blood samples from the same subjects were also taken exactly the same day of saliva collection in order to compare the pattern of change in saliva and blood (results are not included in this report).

It can be seen from data given in **Figure 1**, that a mean 20-25% reduction in salivary glucose has occurred during the first 10 days of Ramadan fasting followed by a less reduction in the next ten days and, finally, another rise by the 29^{th} day of Ramadan fasting. Rise in salivary glucose during the last 10 days of Ramadan did not, however, reach the normal glucose level as compared to the non-fast volenteers. Examination of blood samples of each volenteer showed some similar alternations. However, the results need more investigations and, therefore, are not presented in this paper. There are only a few reports about blood glucose variations due to fasting during Ramadan [12,13]. In the case of blood glucose, reduction during the first 10 days of Ramadan could almost be compensated by the middle of the month due to gluconeogenesis. In saliva, however, maintenance of glucose concentration in low level compared to blood glucose, suggests that gluconeogensis provides glucose mostly for leveling the blood glucose. This is an interesting phenomema bearing in mind that the function of glucose in saliva is not as critical as it is in blood.

5. CONCLUSIONS

Normal salivary function is considered to be critical for the maintenance of healthy oral mucosa [4-6]. Oral fluids provide an easily available non-invasive for the diagnosis of a wide range of diseases and clinical situations. The effect of Ramadan fasting on some blood factors such as thyroid hormons [14,15], plasma lipoproteins [16], serum glucose and many other laboratory findings [11-13] have been reported. However, according to our literature survey, saliva has received less attention in this regard. The laboratory findings reported in this research, indicate that the glucose concentration in saliva is decreased during fasting, mainly at the beginning of the month.

It was shown that about 25 ± 2% reduction in the first 10 days was followed by a rise (17 ± 2% reduction compared to the non-fasting state) in the next ten days and finally another rise in the last 10 days of the month. According to these results, it was concluded that although fasting during Ramadan affected salivary glucose concentration, it had no impact on the glucose function in saliva of healthy people. As concentration of glucose in saliva is highly dependent on the food intake and does not play a crucial role in carbohydrate metabolism [17, 18], we concluded that reduction in salivary glucose during fasting is not accompanied by serious health risks.

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Vol.2, No.7, 773-780 (2010)
doi:10.4236/health.2010.27117

Health

Physical and psychological condition of senior people in a residential care facility. The effects of an aerobic training

Emanuela Rabaglietti[1]*, Liubicich Monica Emma[2], Ciairano Silvia[1]

[1]Department of Psychology, Laboratory of Developmental Psychology, University of TorinoVia Verdi 10, Turin, Italy;
[*]Corresponding Author: emanuela.rabaglietti@unito.it
[2]SUISM–Scuola Universitaria Interfacoltà di Scienze Motorie, Centro Ricerche in Scienze Motorie e Sportive, University of Turin, Turin, Italy

Received 31 December 2009; revised 18 January 2010; accepted 20 January 2009.

ABSTRACT

The present study is aimed at investigating the changes between pre-test and post-test, after having introduced an aerobic programme of physical activity (one session, each of 45' per week for 15 weeks, over a span of roughly four months, delivered by specially trained instructor), in the psychological situation (perception of general health, feelings of geriatric depression) and physical condition (activities of daily living, physical performance in terms of balance and gait, weight, and body mass index–BMI) of a group of senior citizens slightly compromised at Mini Mental State Examination (MMSE: median 23) and living in a residential care facility in northern Italy. The 36-Item Short Form Health Survey Questionnaire (SF-36), the Geriatric Depression Scale (GDS), the Italian short version of Barthel's Index of Activities of Daily Living (BADL), the Tinetti Assessment Tool were administered to 18 old men and women (median age 85.50 years). The findings (we used non-parametric statistical techniques) showed that: 1) general health perception, feelings of depression, and activities of daily were stable; 2) general physical performance, and particularly the balance, increased between pre-test and post-test, while weight, BMI and waist circumference decreased. These results underline the importance, especially in the case of women, of participating at an aerobic training delivered by experienced instructors for the physical condition of senior citizens, also when they are slightly cognitively compromised.

Keywords: Senior People; Training; Physical and Psychological Condition

1. INTRODUCTION

The participation at training of physical activity in very old people living in residential care facilities may have some effects in terms of the potential positive consequences for the general physical and psychological conditions. On its turn some improvement in the general physical and psychological conditions may enhance the global quality of life and may contribute to the maintenance of cognitive and physical skills and autonomy.

At increasing age we usually observe a progressive decreasing of physical skills [1], which is related to physiological changes [2]. However, at the same age we found great individual differences in the amount of daily living autonomy [3]. Furthermore, at very old ages, the biological potential of the individuals is even further weakened, leading to different forms of frailties [4,5], which contribute to make older people much vulnerable and less apt at mastering the tasks of daily living.

Among the senior people, frailty is a heterogeneous syndrome that includes aspects related to physical functioning, such as walking, balance and strength, metabolic aspects as the body max index, and psychological and cognitive aspects [6,7]. Some recent studies underlined that in elderly people there is a relationship between decreasing of physical abilities and both cognitive decline [8] and modification of the body mass index [9]. More specifically, a increase of the body mass index was found positively related to the development of physical disabilities, especially among women, while the maintenance of a correct body weight was related with high levels of physical functioning, and consequently with a great autonomy in mastering the tasks of daily living [10]. The study by Bohannon and colleagues [11] underlined, in a sample of older women aged 74.9 years and living in residential care facilities, a strong relationship between increase in the body mass index and decrease of the physical ability linked to walking. The decrease of such abilities, which is expressed by deficit in strength, resistance and balance, is determinant for the loss of autonomy, leading the older people to dependence in facing the basic activities of daily living [12,13].

Generally speaking, the condition of frailty, particularly in senior citizens who are institutionalized for long period, is often associated to episodes of fall that represent a risk factor not only for the physical disability but also for the potential negative psychological consequences [14,15]. In fact, the loss of self confidence and the fear of falling may accelerate the functional decline and may induce depression [16] and/or isolation, particularly among women and the most old people [17-20]. Besides, the elderly people who repeatedly fall are not only at risk of death, they tend to develop more rapidly than their mates deficits in the skills of successfully mastering basic daily living activities [21].

The benefits of a regular physical activity for the psychological and physical condition of senior citizens have been already widely acknowledged. Among the others, the meta-analysis by McCauley [22] showed that about 69% of researches published in this field found a positive association between physical activity and the psychological wellbeing of the elderly people. This positive association was confirmed by the meta-analysis by Kramer *et al.* [1] that paid attention particularly at the positive effects of aerobic training on the cognitive functioning of the senior people. Also the more recent study by Wang and colleagues [9] showed that a increase in physical activity is associated with a decrease in the risk of onset of dementia. Furthermore, some studies on population [3], showed that the association between physical activity and health is especially strong in women and most old groups of people. In general, physical activity contributes to the wellbeing of the person fulfilling a preventive action against the onset of different pathologies, and among these pathologies especially physical disability [23].

According to the indications of the American College of Sports Medicine [24], the programs of physical activity addressed at the elderly people in the residential care facilities are aimed at preserving the abilities of daily living, which are necessary for conserving a certain independence as long as possible, at delaying the onset of chronic pathologies and at promoting the wellbeing of the older people, by offering opportunities of social interactions. Different typologies of exercises were showed able to improving the physical abilities and the quality of life of the senior citizens [25]. Among these exercises particularly effective seem those that require a multilateral approach at physical activity, by proposing specific exercises for joint mobility, balance, strength and resistance, and respecting the individual level of ability.

Despite the relevance of investigating these issues in different samples, the great majority of the studies concentrated on senior citizens aged from 65 and 75 years [14,22]. Given the increasing life expectancy in all the western society [26] we think that it is relevant to start to investigate much older samples.

The present longitudinal study represent the continuation of some previous researches [27-29] that demonstrated the positive effect of an aerobic programme of physical activity in an Italian sample of senior citizens in residential care facilities on positive self-perception and the perception that health limits physical activities.

In the present study we further extended our interest focusing on a very old group of people and investigating also measures of physical wellbeing and performance. Specifically, we looked at a group of elderly people, who lived in a residential care facility and who were shown slightly impaired at the Mini Mental State Examination (MMSE), to describe the changes between pre-test and post-test in relation to the participation at an aerobic programme of physical activity, on psychological aspects (such as general health perception and feelings of depression), abilities of daily living, and physical aspects (in terms of balance and gait, weight, body mass index-BMI-and waist circumference).

In relation to the phase of development of very old ages, we have to acknowledge that it is usually characterized by a general slowness of the processes of change, particularly with respect to the self evaluation of physical and psychological condition that may need some time for being appreciated [30]. Besides, also a slight cognitive impairment, as that showed by our participants, may even further slow the appreciation of eventual changes in self-perception because of crystallization of the previous self-evaluation. Thus, we expected that at very old ages the participation at training of aerobic physical activity contributes to modify more the aspects connected to the physical condition, than the psychological dimension such as general health perception, depressive feelings and basic activities of daily living. Besides, we have to consider that very old people living in residential care facilities may have a rather unfavorable balance in terms of resources and/or ties they can count on: they are all institutionalized from long time and therefore they may have lost much of the resources on which usually people count on, as the small daily habits of living independently at home and the daily relationships with the external context.

2. METHODS

2.1. Study Design

The intervention was introduced in a residential care facility in northern Italy. It is a private structure but linked to the public health service with a funding agreement. This facility houses both self-sufficient (that is, they can still walk, eat and go in the bathroom by themselves) and dependent (requiring assistance with essential activities of daily living) senior citizens; it also provides the self-sufficient guests a daily physiotherapy session.

2.2. Description of the Intervention

The intervention consisted of one session (lasting 45' each) per week for 15 weeks, over a span of roughly four months. The intervention was addressed to a group of self-sufficient elderly people living in a residential care facility. The sessions were conducted by one instructor, she had university degree in physical education and sport-related fields and was specialised in physical fitness training for older people [28].

The aerobic programme aimed to achieve five main goals:

1) to improve respiratory function through deep breathing techniques;

2) to promote the awareness of incorrect or compensative posture and to learn to modify these problems on their own;

3) to execute movements addressed to the various joints, trying to reach the maximum possible exertion, without exceeding personal limitations;

4) to reach a correct perception of one's body in various conditions of static and dynamic equilibrium;

5) to strength interpersonal relationships and rediscover the joys of playing and using abilities that may have been perceived as lost, by exercising in pairs or small groups.

The intervention was tailored to engage gradually and to interest the elderly people in a variety of different kinds of activities, by using both conventional and unconventional instruments (such as stools, sticks, clubs, hoops, balloons, foam balls, towels, paper cups, pins, bowls, paper tissues, scarves, and trays) and by stressing playful qualities. Besides, the instructor was daily informed by the other personnel about the condition of each older person, also in terms of minor physical problems, and they avoided asking for potentially dangerous movements. Furthermore, special care was taken to provide older people with plenty of time to execute each movement, avoiding activities that could have been perceived as too intense, embarrassing, or difficult.

2.3. The Participants

The selection of the senior citizens was conducted by the director of the residential care facility, who is a trained physician, from among all the elderly people living in the facility. The two criteria for inclusion were: 1) self-sufficiency (defined as above), and 2) absence of serious chronic and/or acute diseases, which was verified directly by the researchers. The Mini Mental Test Examination (MMSE) was used to evaluate cognitive capacity [7]: the participants were assessed slightly cognitively impaired since the median score was 23 and the range scored between 18 and 24.

The participants were informed that participation in the study was voluntary and confidential. All the selected senior people agreed to participate and gave informed consent, in accordance with Italian law and the Association of Italian Psychologists' ethical code [31].

The group of participants comprised 18 senior people, of whom 6 (33%) were men and 12 (67%) women. The median age was 85.50 years (Mean = 85.86, SD = 5.64; range 74-96). All the participants lived in the residential care facility permanently. All except one, who was born in the centre of Italy, were from the same region where the facility is located. With regard to marriage status the majority (N = 11, 60%) were widows, the 28% (N = 5) were married and the others had never been married (N = 1), or were divorced (N = 1). In terms of education, two levels were considered: 'low', corresponding to compulsory education (primary and secondary school) and 'high', corresponding to additional non-compulsory education (including high school and university). The average level of education for both men and women in the sample was similar at the national statistics of population for age-matched [19,32]. Among participants, 67% percent (N = 12) had received compulsory education compared to about 70 percent in the national population. Former occupations were dichotomised in manual (N = 12, 67%) and non-manual labour (N = 6, 33%). This ratio closely reflects the national population. The majority (N = 12, 67%) had never participated in organised exercise or sport activities. Of those who had, the preferred sports were bowls, gymnastic, soccer, and walking.

The administration of the whole set of instruments at the pre-test and post-test was very long (about 3-4 hours for participant including both psychological and physical measures). Thus, we did not succeed in collecting the complete information in 6 participants (1 man and 5 women) with respect to the physical measures and in 1 women with respect to the questionnaires because these elderly people cannot participate at all the administration sessions during the weeks planned for pre-test and/or post-test for different reasons, mainly because of suffering slight indispositions and/or having became acute again of small affliction. However, we did not find any difference between this subgroup of participants with not complete information and their mates nor with respect to the socio-demographic information, neither with respect to the study variables that we were able to collect.

2.4. Procedure

From the Italian version of the 36-Item Short Form Health Survey Questionnaire (SF-36; [33,34]), in the present study we used only the question that investigates the general perception of health (Range of possible answers: 1 bad-5 excellent).

Besides the senior citizens were administered the Italian short version of the Geriatric Depression Scale (GDS; [15,35]). This questionnaire consists of 15 items referring to seven common characteristics of depression in older life: somatic concern, lowered affect, cognitive impairment, feelings of discrimination, impaired motivation, lack of future orientation and of self-esteem. Each question has dichotomous answers: 0 no, 1 yes (presence of a characteristic in the area of depression) for a possible range 0-15. For total scores between 0 and 5 the level of depression is usually considered normal, while total scores higher than 5 express different levels of depression. Among our participants, the Cronbach's alpha was good at both waves (pre-test = 0.70, post-test = 0.90).

We also used the Italian short version of Index of Basic Activities of Daily Living (BADL; [23,36]). This questionnaire is a well-validated measure of functional outcomes and it includes self-evaluation of independence and/or need of some help in mobility and self-care abilities in 6 areas such as feeding, bathing, dressing, walking, and bowel and bladder control. Each question has dichotomous answers: 0 by oneself, no help, 1 with help for a possible range 0-6. The Cronbach's alpha was very good at both waves (pre-test = 0.80, post-test = 0.88).

We also administered the Tinetti Assessment Tool [37] for evaluating gait and balance in older people. This test is a task performance exam, which quantifies the motor performance and identifies the subjects at risk of falling by two scales: the Tinetti Balance Scale o BPOMA, which assesses the characteristics of sitting station, postural passages, and erect station, and the Tinetti Gait Scale o GPOMA, which assesses the characteristics of walking.

Finally, all the participants were weighed at pre-test and post-test and we calculated BMI by the way of an electronic balance.

All these measures were collected by specially trained researchers.

2.5. Strategy of Analysis

We used Wilcoxon test for dependent samples for analyzing the presence of differences between pre-test and post-test for the whole set of measures and for the whole group of participants (**Table 1**).

Besides, we used Wilcoxon test for dependent sample for analyzing the presence of differences between pre-test and post-test separately in the sub-groups of men and women (data reported directly in the text). For evaluating the effectiveness of the intervention we calculated the width of a not parametric effect size, which is more appropriate with very little samples than other kinds of measures [38]. We also used Mann-Whitney test for independent samples for analyzing the presence of differences between men and women separately in each time (**Table 2**).

Finally, in all the cases we describe our data using both median, which may be more adequate than other descriptive measures in very little sample, and mean and standard deviation.

3. RESULTS

As we hypothesised we did not find any significant changes between pre-test and post-test with respect to psychological aspects, such as general health perception, depression, and activities of daily living as they were perceived by the older people (**Table 1**). However, general health perception showed some tendency to increase between pre-test and post-test.

With respect to the physical performance as it is measured by Tinetti test, we found a relevant increase of the balance between pre-test and post-test, while the gait was stable.

In all the above mentioned aspects such as psychological characteristics and physical performance, we did not find any statistical differences between men and women in both waves (these data are available at the first author). However, the effect of the intervention on the balance (measured by the Tinetti Balance Scale) was greater for women than for men. For women: Pre-test: median = 10.0, M = 10.43, D.S. = 3.10; Post-test: median = 13.00, M = 13.43, D.S. = 2.57; Z = –2.375, $p < 0.02$, ES = 0.63. For men: Pre-test: median = 10.0, M = 10.40, D.S. = 1.67; Post-test: median = 12.00, M = 9.40, D.S. = 5.18; Z = –0.137, p = 0.89, ES = 0.04.

In relation to the physical condition we found significant and positive changes between pre-test and post-test for every aspect considered in the study: in general, participants decreased their weight, BMI and also waist circumference (**Table 1**).

With respect to the physical condition, as it was reasonable to expect, we also found in each wave separately significant gender differences (**Table 2**): women in general weighted less and they had lower BMI and waist

Table 1. Wilcoxon test for dependent samples on psychological and physical indicators of adjustment—difference between pre and post-test.

Variable	Z	N	Sig.[1]	Effect size (ES)	Median		Means (St. dev.)	
					Pre-test	Post-test	Pre-test	Post-test
General Health Perception	–1.134	18	0.26	0.19	2	2	2.10 (0.64)	2.35 (0.61)
GDS	–0.114	18	0.91	0.02	3	4	4.65 (3.72)	5.12 (4.49)
BADL	–0.287	18	0.77	0.05	5	6	4.47 (1.84)	4.59 (2.03)
Tinetti Balance	–1.663	12	0.09	0.34	11	12.50	10.42 (2.50)	11.75 (4.20)
Tinetti Gait	–1.000	12	0.32	0.20	6.50	6.50	5.92 (3.09)	5.83 (3.21)
Weight	–2.001	12	0.04	0.41	66.30	61.60	65.28 (12.61)	63.79 (12.83)
BMI	–2.353	12	0.02	0.50	24.21	23.40	24.50 (3.09)	23.93 (3.25)
Waist circumference	–2.689	12	0.007	0.57	94.50	93.50	93.75 (14.09)	91.75 (14.02)

[1]We considered till $p < 0.10$ because of the very little sample size.

Table 2. Mann-Whitney test for independent samples on psychological and physical indicators of adjustment—significant gender differences.

Variable	U	N	Sig.[2]	Men		Women	
				Median	Means (St. dev.)	Median	Means (St. dev.)
Weight pre-test	3.00	12	0.02	76.30	74.96 (9.51)	54.60	58.37 (9.87)
Weight post-test	3.00	12	0.02	76.10	73.94 (9.37)	54.70	56.54 (9.80)
BMI pre-test	6.50	12	0.07	26.87	26.55 (2.65)	23.32	23.04 (2.61)
BMI post-test	3.00	12	0.02	25.90	26.18 (2.47)	22.60	22.31 (2.83)
Waist circumference pre	–2.353	12	0.02	103.00	105.00 (7.75)	90.00	85.71 (11.97)
Waist circumference post	–2.689	12	0.007	103.00	103.4 (7.67)	86.00	83.43 (11.28)

[2]We considered till $p < 0.10$ because of the very little sample size.

circumference than men. Furthermore, the effect of the intervention on two out of three aspects of the physical condition considered in the present study (weight and BMI) was greater for women than for men. For weight: Women Z = –1.690, $p < 0.09$, ES = 0.45; Men Z = –1.084, p = 0.28, ES = 0.34. For BMI: Women Z = –2.028, $p < 0.04$, ES = 0.54; Men Z = –1.214, p = 0.283, ES = 0.38.

However the effect of the intervention was similar in women and men with respect to waist circumference. For Waist circumference: Women Z = –2.060, $p < 0.04$, ES = 0.55; Men Z = –1.857, $p < 0.06$, ES = 0.59.

Generally speaking the intervention was showed to have medium-large effect size with respect to the physical performance and condition.

4. DISCUSSION AND CONCLUSIONS

This study was aimed at investigating the effect of the participation at an aerobic physical training in senior citizens who were slightly cognitively impaired and who lived in a residential care facility. More specifically the study was aimed at investigating the changes between pre-test and post-test with respect to psychological and self-report characteristics, as the perception of health in general, depression and abilities of daily living, physical performance, in terms of balance and gait, and the physical condition, in terms of weight, BMI and waist circumference.

As expected we did not find any change in general health perception and depressive feelings. We also did not find any change in the perception of abilities of daily living in the elderly people. For certain aspects these findings seemed to confirm what we have already underlined in a previous study [29]. In this previous study we found that to change the general health perception and the negative self perception of senior people living in residential care facilities is much more difficult than to change other aspects, as the positive self perception and the perception that health may limit physical activity. However, we need also to reflect on the specific characteristics of the participants at the present study. At first, the participants at the present study already suffered for some limitations at their autonomy from the beginning: three of them used some aids. Considering this condition, the fact that they did not get worse along time may be considered a success of the intervention. However, we lack a comparable control group in order to be able to claim this phenomenon as a positive effect of the participation at the training of physical activity. At second, contrarily to what was shown in some previous studies about the positive effect of physical activity on depression in senior citizens [39], we found that depression was stable between pre-test and post-test and it was even slightly higher at the post-test than at the pre-test. We certainly need to further investigate this important point. However, we also have to consider that the age of the participants at the present study is much higher than the ages of the participants at the above mentioned studies.

We found greater evidence of the effectiveness of the intervention on the physical performance and the physical condition than on the psychological and self report aspects. At first we found a relevant improvement of the physical performance in terms of balance. This finding confirmed the study by Harada and collegues [6] on the possibility of improving the physical functioning of institutionalized elderly people by the way of specific training programs. With respect to the fact that the intervention seemed more effective in changing the balance rather than the gait of the senior people, our findings suggested that the balance might be easier to change in short period, while long period may be necessary for changing the gait aspects. At second we found that the intervention was very powerful in ameliorating the physical condition of the senior citizens, contributing at decreasing their weight, BMI, and waist circumference. This finding extended the results of a series of previous studies [8,29,39,40], which underlined different potential benefits of the participation at physical training for the elderly people, in a very old group of participants with slight cognitive impairment. We think that this finding is very comforting, because it showed that a moderate but structured and regular physical training (that lasted for 15 weeks only) may promote a decrease in the body weight, and consequently in the BMI, contributing to a great improvement of the functional condition of senior citizens who are living a condition of general frailty. That is a condition, as it is meant by Fried and Walston [41-43], that may put these senior people at great risk of general disability and co morbidity. At third, we found the participation at the training of physical activity was particularly effective in the case of women. This also is a relevant finding considering that the life expectancy of women is currently increasing much more than that of men [26].

This study has several limitations and among the most important there are the little sample size and the lack of an equivalent control group. These limitations do not allow us to generalize our findings to different situations and populations. Furthermore, although we are aware that the objective clinical parameters as bloody pressure and biochemical texts may be very difficult to change in short periods in senior citizens and also that some previous studies doubted that these clinical parameters may be assumed as efficient predictors of the general health condition of the older people [14], yet we have to admit the lack of these measures in our study.

Despite these and other limitations, our study gives some interesting cues. At first it showed the benefits of the participation at a training of physical activity for institutionalized senior citizens. This represents both a confirmation and an extension of what we already showed in some previous studies that focused only on self-report measures [28,29]. At second it underlined that also in critical condition, as that of the elderly people living in residential care facility and slightly cognitively impaired, the introduction of a relatively simple training may have positive effects on the individual functioning in short time.

5. ACKNOWLEDGEMENTS

The third author wants to acknowledge the contribution of Regione Piemonte, Direzione Sanità-Settore Igiene e Sanità Pubblica at this study.

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Vol.2, No.7, 781-788 (2010)
doi:10.4236/health.2010.27118

Health

Internal environment in cancer patients and proposal that carcinogenesis is adaptive response of glycolysis to overcome adverse internal conditions

Mayumi Watanabe[1], Kenya Miyajima[2], Ittoku Matsui[2], Chikako Tomiyama-Miyaji[3], Eisuke Kainuma[1], Masashi Inoue[1], Hiroaki Matsumoto[1], Yuh Kuwano[1], Toru Abo[1]*

[1]Department of Immunology, Niigata University School of Medicine, Niigata, Japan;
[*]Corresponding Author: immunol2@med.niigata-u.ac.jp
[2]Yushima-Shimizuzaka Clinic, Tokyo, Japan
[3]School of Health Sciences, Faculty of Medicine, Niigata University, Niigata, Japan

Received 4 March 2010; revised 16 March 2010; accepted 20 March 2010.

ABSTRACT

In a series of our recent studies, stress was found to induce simultaneously hypothermia and hyperglycemia. These conditions are beneficial to obtain prompt force which depends on the glycolysis pathway and to escape emergency. Since we have noticed that such conditions resemble the internal environment seen in some cancer patients, it was investigated whether such conditions were accompanied with other patients. We selected patients with early and advanced cancer. Body temperature and other parameters including blood gas contents were examined. A difference was seen in body temperature, namely, many patients showed hypothermia, irrespective of cancer stages. Further characterization of other parameters showed that hypothermia and hyperglycemia existed in many patients. They had immunosuppressive state and anemia. Blood gas analysis showed that oxygen contents were low and carbon dioxide contents were high in patients. These results suggest a possibility that the internal environment seen in patients is responsible to induce onset of disease and to maintain their cell growth, because cancer cells have an energy system of predominant glycolysis. Although hypothermia, hypoxia and hyperglycemia are important to activate the glycolysis pathway and to escape from emergency, such responses suppress the mitochondrial pathway for long span and may result in carcinogenesis.

Keywords: Cancer; Hypothermia; Hypoxia; Hyperglycemia; Glycolysis; Mitochondria

1. INTRODUCTION

Many investigators and clinicians have felt that cancer might be a systemic disease although tumor masses are primarily present at local sites. If this is the case, we have to consider specific, common internal environment in cancer patients. In the course of the analysis of many parameters in cancer patients, we noticed that many cancer patients showed simultaneous hypothermia and hyperglycemia.

In light of these findings, we then investigated the internal environment in relation to stress-associated responses [1,2]. Of interest was that both hypothermia and hyperglycemia were induced by stress. Such an internal environment might be beneficial for humans and animals to escape emergencies [3]. Namely, prompt output of force by white muscle fibers depends on the energy production system of glycolysis. Although the efficiency of energy production is low (2 ATP/glucose) in the glycolysis pathway, the ATP synthesis in this system is much quicker (× 100) than that of the mitochondrial system (× 1) [4].

In a short span of time, hypothermia and hyperglycemia are therefore good conditions for escape from stress or emergencies. However, these conditions are not appropriate for energy production of the mitochondrial pathway (*i.e.*, oxidative phosphorylation). Many patients with hypothermia and hyperglycemia suffer from general fatigue, emaciated conditions, diabetic disease, etc.

In the present study, we investigated the internal environment in cancer patients in detail and herein propose on adaptation theory, namely, that the onset of cancer is a phenomenon of a glycolytic adaptation response by living beings to overcome deteriorated internal conditions in the body. Cumulative evidence has shown that cancer cells have a shift of glucose metabolism from oxidative phosphorylation to glycolysis, eventually resulting in few or defective mitochondria in the cytoplasm [5-7]. Although many investigators have considered that carcinogens such as ultraviolet rays, food additives, air pollution, etc. [8-12], induce multiple mutation steps in proto-oncogenes, there is an alternative possibility that such mutation is a process of glycolytic adaptation by living beings, namely, cancer cells. Hypothermia, hypoxia and hyperglycemia, which are induced by continuous stress (due to the lifestyle in patients), might be important factors which induce the adaptive response.

2. MATERIALS AND METHODS

2.1. Subjects

Patients with early or advanced cancer were first examined as to body temperature (n = 28). They were 54.3 ± 8.0 years old. Age-matched healthy controls (n = 27), 45.8 ± 11.0 years of age, were also examined.

For detailed analysis of many parameters other than body temperature, patients with advanced cancer (n = 13) were then selected (**Table 1**). Details of the cancer patients are listed in the table, including sex and age (52.1 ± 8.7 years old). At the time of analysis, these patients were receiving neither chemotherapy nor irradiation therapy. Age-matched healthy controls (n = 11), 46.7 ± 10.0 of age, were also examined.

Table 1. Patients with Advanced Cancer.

Case	Type of Cancer	Sex	Age
1	chronic myelogenic leukemia	F	68
2	ovarian cancer	F	56
3	brain cancer	M	50
4	stomach cancer	F	63
5	parotid gland cance	F	48
6	rectum cancer, metastasis to lung	M	58
7	uterus cervical cancer	F	36
8	rectum cancer, metastasis to lung	M	45
9	bladder cancer	M	52
10	lung cancer	M	48
11	breast cancer	F	56
12	malignant lymphoma	M	42
13	stomach cancer	M	55

Informed consent was obtained from all subjects.

2.2. Parameters Tested

Blood for the analysis was obtained from a vein. Blood glucose was measured by Precision Xtra TM (Abott Japan Co., Ltd., Chiba, Japan). Venous blood analysis of lactate and of the levels of pH, O_2 and CO_2 was also performed using i-STAT 300F (i-STAT Corporation, NJ, USA).

To analyze the hematological parameters, leukocyte counts of fresh venous blood were determined by hemocytemeter and were stained by the Giemsa method. The contents of hemoglobin (Hb) and others in the blood were measured by Sodium Lauryl Sulfate (SLS)-Hb methods and hematocytemeter, respectively.

2.3. Statistical Analysis

The difference between the values was determined by Student's *t*-test, Mann-Whitney's U test and Welch's *t*-test.

3. RESULTS

3.1. Hypothermia and Hyperglycemia Seen in Cancer Patients

Twenty-eight patients with early or advanced cancer and twenty-seven healthy subjects were examined as to body temperature (**Figure 1(a)**). It was found that there were many persons with hypothermia (36.1 ± 0.5˚C) among cancer patients in comparison with healthy persons (36.6 ± 0.4˚C), the difference being statistically significant ($p < 0.01$).

We then analyzed many parameters in patients with advanced cancer (n = 13) and age-matched controls (n = 11) (**Figure 1(b)**). Hypothermia was confirmed in these patients with advanced cancer (35.9 ± 0.5˚C) in comparison with controls (36.5 ± 0.3˚C). In addition to hypothermia, hyperglycemia was also detected in cancer patients (125.3 ± 28.5 mg/dL) in comparison with controls (106.3 ± 11.1 mg/dL).

Since stress-associated responses simultaneously induce hypothermia and hyperglycemia, other stress-associated parameters were also examined in this experiment (**Figure 1(b)**, bottom). Although there was a tendency that some patients with advanced cancer had a high pulse rate (sympathetic nerve activation) and a high level of lactate, these were not common to all patients ($p > 0.05$ in both pulse and lactate).

3.2. Immunosuppressive States and Anemia in Cancer Patients

Immunoparameters were examined in cancer patients and

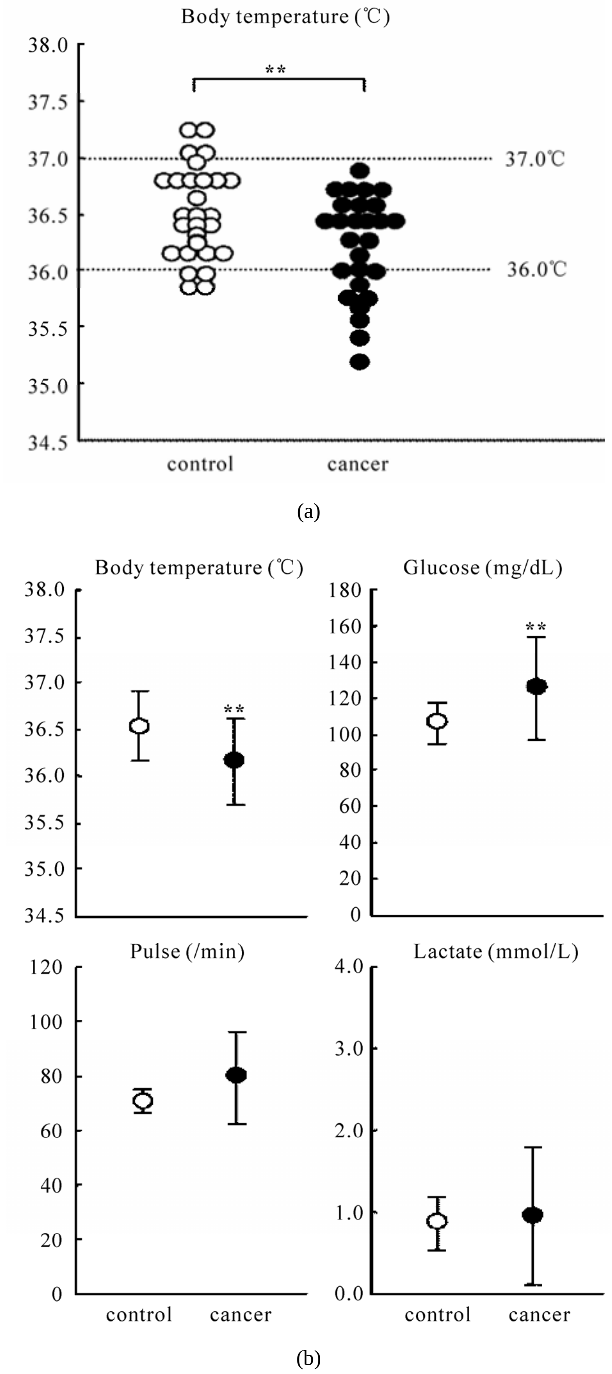


Figure 1. (a) Comparison of body temperature and other parameters between healthy controls and cancer patients. (a). Body temperature, (b). Further analysis of body temperature and others. In experiment (a), healthy controls (n = 27) and cancer patients (n = 28) were examined. In experiment (b), healthy controls (n = 11) and patients with advanced cancer (n = 13) were examined. In addition to body temperature, the levels of glucose and lactate and the pulse rate were examined in this experiment. Body temperature was measured in the axilla for 3 min. **p < 0.01.

healthy controls (**Figure 2(a)**). The total number of white blood cells (WBC) was lower in patients (4691 ± 1769 /μL) than in controls (6190 ± 1088 /μL) ($p < 0.05$). When the ratio of WBC (leukocyte) subsets was enumerated, the ratio of granulocytes was found to be high, while that of lymphocytes was low ($p < 0.01$). The ratio of monocytes was comparable in patients and controls. By calculation, the absolute number of leukocyte subsets was determined. It was found that the most prominent distinction was in lymphocytes, namely, the number of lymphocytes in patients (1334 ± 476 /μL) was extremely low in comparison with the number in controls (2387 ± 538 /μL) ($p < 0.01$). The decrease in the number of leukocytes seen in patients was found to be due to the decrease in the number of lymphocytes. The ratio and number of monocytes were comparable in controls and cancer patients.

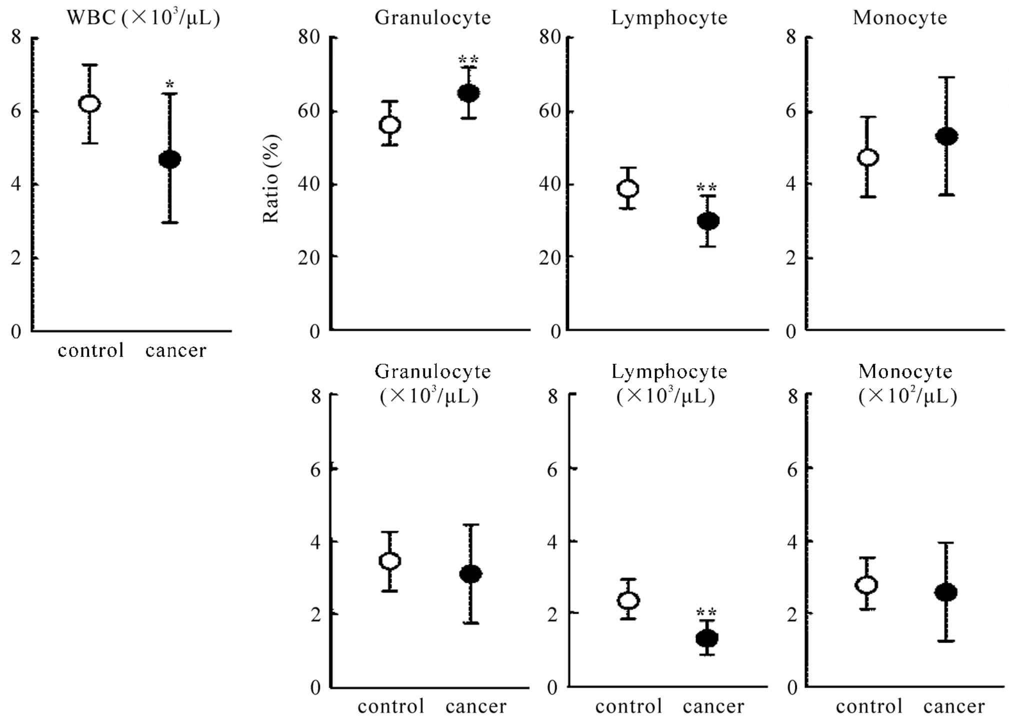
The level of red blood cells (RBC) and related parameters was then examined (**Figure 2(b)**). In addition to the decrease in the number of WBC, the number of RBC was found to decrease in cancer patients ($407 \pm 47 \times 10^4$/μL) in comparison with controls ($446 \pm 39 \times 10^4$/μL) ($p < 0.05$). The level of Hb was lower in patients (11.9 ± 1.7 g/dL) than in controls (13.9 ± 1.3 g/dL). The level of hematocrit (Ht) was also lower in patients (37.6 ± 4.1%) than in controls (42.0 ± 3.5%) ($p < 0.01$). Other parameters of RBC, namely, mean red cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were all comparable between controls and patients ($p > 0.05$). This was also the case for the number of platelets.

3.3. Blood Gas Analysis in Cancer Patients

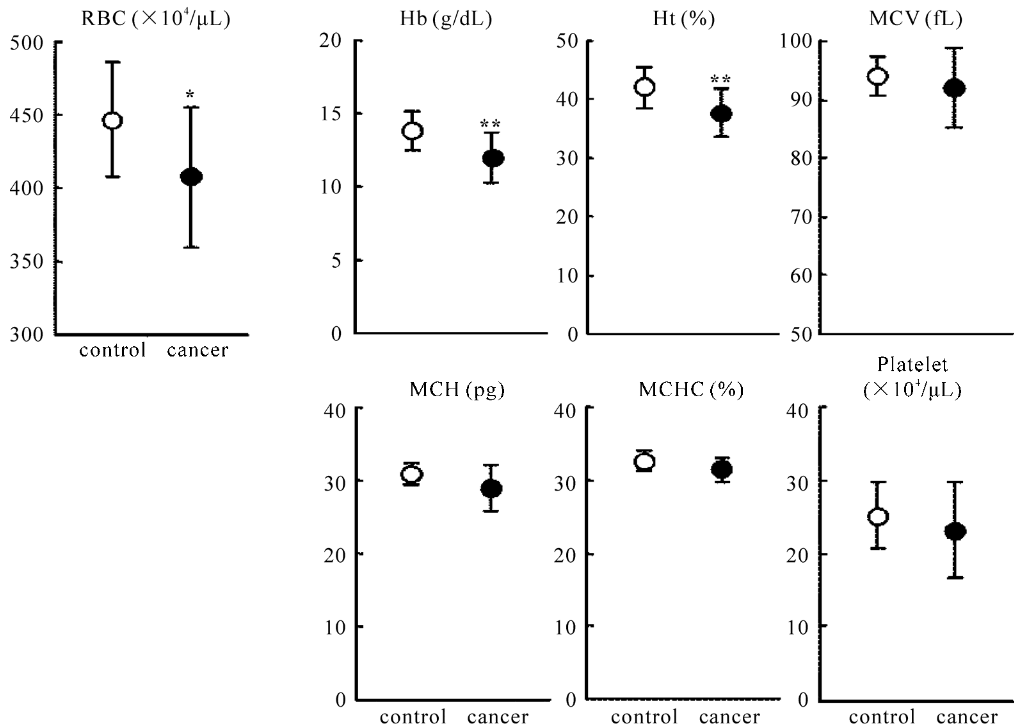
It is conceivable that hypothermia and anemia seen in patients with advanced cancer may influence the parameters as shown by blood gas analysis. Therefore, such analysis using venous blood was conducted (**Figure 3**). It was demonstrated that blood pH was lower in patients (7.36 ± 0.03) than in controls (7.40 ± 0.03) ($p < 0.05$). The major factors influencing blood pH are known to be the levels of O_2 and CO_2 contents. Indeed, the levels of PO_2 (mmHg) and SO_2 (%) were found to be extremely low in patients ($p < 0.01$, $p < 0.05$, respectively). On the other hand, the levels TCO_2 (mmol/L) and PCO_2 (mmHg) tended to be high in patients. BEecf (mmol/L), which shows a base excess in extracellular fluids, was slightly high in cancer patients.

4. DISCUSSION

We herein demonstrated that many cancer patients had hypothermia, hypoxia and hyperglycemia simultaneously. Immunosuppressive states, showing granulocytosis and



(a)



(b)

Figure 2. Blood cell analysis. (a) Immunoparameters. (b) Analysis of the number of RBC and the levels of Hb and other parameters. In experiment (a), the absolute number of leukocyte subsets was calculated from the data on the number of WBC and the ratio of leukocyte subsets. In experiment (b), Number of RBC and levels of Hb, Ht, MCV, MCH and MCHC, including the number of platelets, were examined * $p < 0.05$, ** $p < 0.01$.

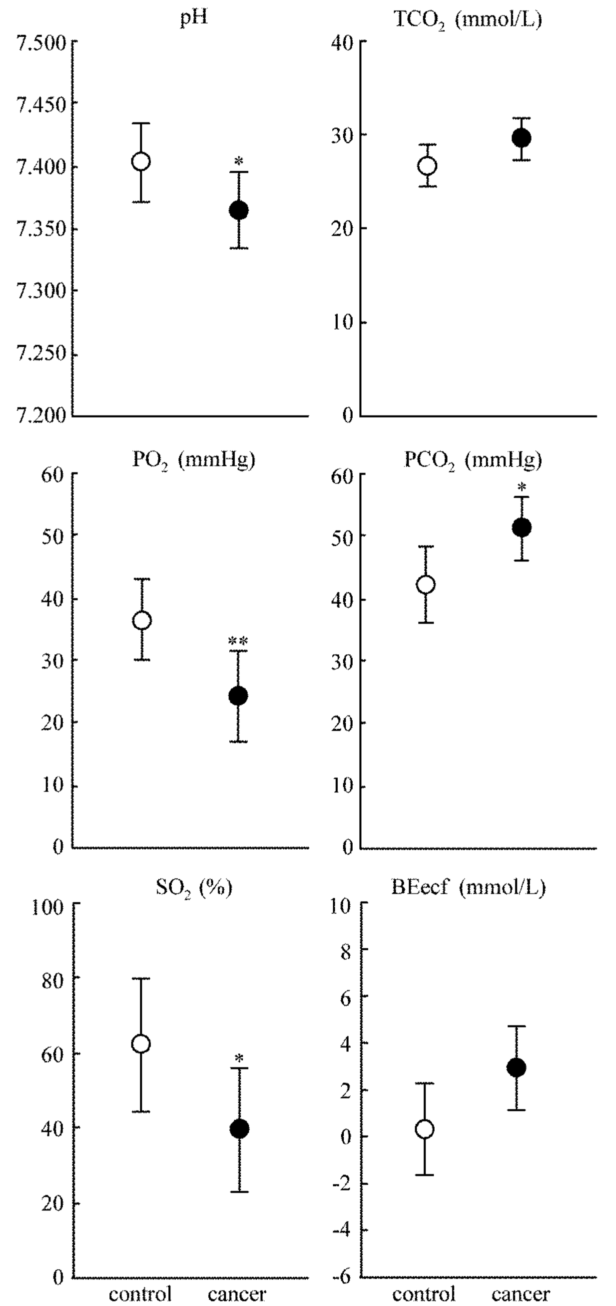


Figure 3. pH and blood gas analysis in healthy controls and cancer patients. Venous blood was used for the analysis. * $p < 0.05$, ** $p < 0.01$.

lymphocytopenia, were also present. Although clinicians are empirically aware of the deteriorated conditions such as hypoxia, immunosuppression and anemia in cancer patients [13-20], as shown by a review of the literature, few studies have been done on the simultaneous identification of all these conditions. We propose the possibility that hypothermia, hypoxia and hyperglycemia are beneficial for stress-exposed persons to escape from emergencies inducing stress for short periods of time, but that such internal environment might become cancer-inducing over a longer period of time. This proposal is based on an understanding of the energy production system comprising the glycolysis and mitochondria pathways [3,4].

It has been speculated that the ancestors of eukaryocytes were generated by a connection between living beings with glycolysis and those with mitochondria at approximately 2 billion years ago [21,22]. Under such situation, eukaryocytes had two energy production methods, namely, the glycolysis and mitochondria pathways (**Table 2**). As shown in this table, the functioning conditions, usage and other characteristics between two pathways are quite different. If we consider the internal environment (*i.e.*, hypoxia and hyperglycemia) seen in cancer patients, these conditions are rather appropriate for the function of glycolysis.

In a recent study, we analyzed in detail the stress-associated conditions in mice exposed to restraint stress [1,2]. Of interest was that such conditions were revealed to be hypothermia and hyperglycemia. In addition, the administration of catecholamines or glycocorticoids also directly induced hypothermia and hyperglycemia [2]. In a short span of time, such internal conditions are beneficial for humans and animals to obtain the prompt force of white muscle fibers via activation of the glycolysis

Table 2. Energy production system.

	Glycolysis Pathway	Mitochondrial Pathway
Site	cytoplasm	mitochondria
Oxgen	– or ±	++
Source	glucose	pyruvic acid (lactate) ketone bodies
Temperature	32-36℃	> 37℃
Usage	cell division prompt force	suppression of cell division continuous force
ATP production	quick (× 100)	slow (× 1)
Efficiency	low (2ATP/glucose)	high (36ATP/glucose)
Cells	sperms cancer cells skin cells bone marrow cells white muscle cells	cardiac muscle cells neurons hepatocytes red muscle cells many other cells

pathway [3]. As a result, such conditions realize the power needed to escape from stressful conditions such as those in emergencies. In other words, the internal conditions of hypothermia and hyperglycemia do not seem to be a failure of our body responses.

If a certain person is exposed to stress for a long time, the internal conditions of hypothermia and hyperglycemia then suppress the mitochondria pathway which produces continuous force and energy for protein synthesis. Such a person might be suffering from general fatigue, emaciated conditions, diabetic disease and other difficulties. This notion seems to be important for understanding the mechanisms involved in the onset of many diseases.

In addition, we propose another possibility of hypothermia, hypoxia and hyperglycemia which may be associated with the onset of malignancy. As shown in **Table 3**, many investigators and clinicians have believed that carcinogens are key factors which induce cancer via the multiple mutation steps of proto-oncogenes [23-27]. However, such carcinogens do not seem to be always present in actual cases. Given this fact, such cases may be rather rare. We propose herein the possibility that the internal environment (*i.e.*, hypoxia and hyperglycemia) induced by continuous stress results in an adaptation response in which normally dividing cells become cancer cells (*i.e.*, living beings with glycolysis). In such cases, stress may be caused by overwork, mental stress, obesity, etc., namely, their lifestyle. We consider that these cases are of higher frequency than those due to carcinogens in carcinogenesis. Immunosuppression and anemia in cancer patients as revealed in the present study might result from such stress via the activation of sympathetic nerves [28]. Interaction of leukocyte subsets with catecholamines or glucocorticoids (hypothalamic-pituitary-adrenal axis) was reported previously [29].

Table 3. Transformation to Cancer Cells.

	Direct Cause	Secondary Response	Frequency
Carcinogens	ultraviolet food additives radiation air pollution other carcinogens	mutation of proto-oncogenes or other genes by carcinogens	Less
Stress from lifestyle	hypothermia hypoxia hyperglycemia	mutation of proto-oncogenes or other genes as adaptation to “living beings of glycolysis”	High

O. Warlburg has reported that cancer cells contained a few mitochondria in the cytoplasma and produce energy mainly by the glycolysis pathway [7]. Recent cumulative evidence also supports this earlier observation [30-35]. The functions of oncogenes are eventually related to not only the system of cell-proliferation but also to the system of energy production. The nature of the predominant function of glycolysis in cancer cells is utilized by PET scans [36,37]. The energy produced by glycolysis is used not only to obtain prompt elicitation but also for cell dividing energy (see **Table 2** again). In other words, the initial stress-associated response of hypothermia, hypoxia and hyperglycemia is estimated as allostasis (*i.e.*, change of the internal environment to overcome stress). However, continuous stress then turns to allostatic load and induces the carcinogenesis as adaptation responses (*i.e.*, break of “homeostasis” in our body). These concepts on “allostasis” were proposed by B. S. McEwen, F. S. Dhabhar and their colleagues [38-41]. Stressful life events were reported to be related to such allostatic load [42,43].

In our recent study using hyperthermia equipment [44, 45], many cancer patients could live in good conditions without further tumor enlargement when they exposed to mild hyperthermia (*i.e.*, the maximum rectum temperature is 38.0ºC for 15-30 min). In some cases, tumor regression resulted from mild hyperthermia [45]. At this time, the values of pH, PO_2, PCO_2 and other factors improved. Immunosuppression and anemia seen in cancer patients were also alleviated. These results suggest that a slight shift of glucose metabolism from the glycolysis pathway to the mitochondria pathway (*i.e.*, oxidative phosphorylation) might be important to cure malignancy. Local, strong hyperthermia (e.g., 42ºC) was not effective and rather acted as severe stress in cancer patients. In other words, systemic improvement of the internal environment is critical to cure malignancies. However, we do not recommend patients to use expensive equipment for hyperthermia. The most important things for spontaneous regression of cancer are as follow: changing harmful lifestyle (e.g., overwork), using hot-water bottle at sleeping time, taking a deep breath several times a day, dietary consideration and control of the fear.

We have herein proposed the possibility that stress-associated conditions are beneficial for humans to escape from emergencies in a short span of time, but that the resulting hypothermia and hyperglycemia act as factors which induce the generation of cancer cells. In other words, the onset of malignancy might be a return to “living beings with glycolysis at 2 billion years ago”. Cancer cells eventually contain only a few mitochondria in the cytoplasm. However, we could not neglect a mitochondrial function in tumor cell growth [46,47].

A hypothesis by J. S. Fang, R. J. Gillies and R. A. Gaten was proposed that evolution of carcinogenesis is an adaptation response to hypoxia and acidosis, showing the interaction of cancer cells and microenvironments in the surrounding tissues [48,49]. At that time, a paracrine signaling between epithelial and stromal cells is known to be important for tumor initiation and progression [50]. We were also able to reveal the systemic, internal environment of hypoxia, acidosis and other conditions in actual cancer patients. However, a further research is required to support our proposal definitely. Such research includes an animal experiment using mice with cancer and a cancer cell culture experiment under conditions of hypothermia, hypoxia and hyperglycemia

5. ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. The authors thank Mrs Yuko Kaneko for preparation of the manuscript and Mr Taiki Hashimoto and Ms Kaori Yamamoto (Yushima-Shimizuzaka clinic) for arrangement of the clinical research.

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Vol.2, No.7, 789-795 (2010)
doi:10.4236/health.2010.27119

Health

Taxol as chemical detoxificant of aflatoxin produced by *aspergillus flavus* isolated from sunflower seed

Narasimhan Banu[1]*, Johnpaul Muthumary[2]

[1]Department of Biotehnology, Vels University, Pallavaram, Chennai, India; *Corresponding Author: banunkl@yahoo.com
[2]Centre for Advanced Studies in Botany, University of Madras, Chennai, India

Received 24 November 2009; revised 10 March 2010; accepted 12 March 2010.

ABSTRACT

Aflatoxins are the potent toxic, mutagenic, heterogenic and carcinogenic metabolites produced by species of *A. flavus* and *A. parasiticus*. In the present study, an attempt has been made to prevent aflatoxin production using an anticancerous drug taxol. Taxol (Paclitaxel) is a well known drug for its anticancerous property mainly to treat breast and ovarian cancers. It was obtained from *Taxus brevifolia* and it was also obtained from the endophytic fungi present in *Taxus brefivolia* [1]. Therefore, this drug is specifically selected to screen its activity on the control of *A. flavus* and AFB1 production at various concentrations. Among the 6 concentrations used, 3 µg of taxol was found to be suitable to control the growth and AFB1 production. The content of AFB1 found at this concentration was 6 ppm by TLC and 6.3 ppm by HPTLC. The complete elimination of AFB1 might require higher concentrations of taxol.

Keywords: Aflatoxins; Taxol; Anticancerous Drug; Liver Cancer

1. INTRODUCTION

Aflatoxins are the potent toxic, mutagenic, heterogenic and carcinogenic metabolites produced by species of *A. flavus* and *A. parasiticus* in food and feed, especially the oil seeds and their products, both at pre and post harvest conditions. Their occurrence in food and feed materials has caused not only health hazards in animals and humans but also economic losses especially to the exporting countries. They are initially classified as human carcinogens by the International Agency on Research in Cancer in 1993 and further epidemiological experimental research continues to show a strong link between aflatoxin exposure and hepato cellular carcinoma (HCC).

Although the prevention of mycotoxin contamination in the field is the main goal of agricultural and food industries, under certain environmental conditions the contamination of various commodities with fungi like *Fusarium, Aspergillus, Alternaria* and *Penicillium* and their mycotoxins are unavoidable for producers. Decontamination/detoxification procedure is useful in order to recuperate mycotoxin contaminated commodities. The ideal decontamination procedure should be easy to use, inexpensive and should not lead to the formation of compound that are still toxic, or may reverse to reform the parent mycotoxin or alter the nutritional and palatability properties of the grain or grain products. The frequent occurrence of aflatoxins in food materials poses a serious threat to the consumers. Therefore, a considerable concern has been shown for prevention of the mycotoxins [2]. Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide with incidence rates highest in geographical regions of Africa and Asia exhibiting climatic similarities of high heat, humidity and poor food storage conditions. Reports have shown that aflatoxins causes primary liver cancer in humans on a worldwide basis [3-5]. The International Agency for Research on Cancer [6] has declared AFB1 to be class I carcinogen, on the basis of animal assays. Hence approaches involving physical [7-9] chemical and biological [10-15] methods have been made to detoxify aflatoxins in food and feedstuffs in the recent past. In India, mycotoxin contamination in food and its control were extensively studied by Bilgrami and his associates [16].

Examination of physico-chemical and biochemical characteristics of AFB1 molecule reveals two important sites for toxicological activity [17]. The first site is the double bond in position c-8, 9, of furo-furan ring. The aflatoxin-DNA and protein interactions, which occur at this site, alter the normal biochemical functions of these macromolecules leading to deleterious effects at the cellular level. The second reactive group is the lactone-ring in the coumarin moiety. The lactone ring is easily hydrolyzed; it is therefore a vulnerable site for degradation. Hence the degradation treatments should be aimed at removing the double bond of the terminal furan ring or in opening the lactone ring. Once the lactone ring is opened, further reactions could occur to alter the binding properties of the terminal furan ring to DNA and proteins.

Structural degradation or inactivation of aflatoxins has been found to be possible by the use of chemicals such as chlorinating agents (sodium hypochlorite, chlorine dioxide and gaseous chlorine), oxidizing agents (H_2O_2, ozone, sodium bisulfite) and hydrolytic agents-acids (organic and inorganic) and alkalies (sodium hydroxide, ammonium hydroxide, potassium hydroxide, etc.). Some of these chemicals are already being used in food industry and are less prone to consumer resistance [18]. However, according to Park and Liang [8], most of these chemicals are impractical and are particularly unsafe because they form toxic residues or damage nutrient content, flavour, odour, color, texture or functional properties of the product.

Among different processes, ammoniation process has been extensively worked out and largely accepted in spite of certain resulting nutritional losses. Liquor ammonia, gaseous ammonia, *in situ* liberation of ammonia by reaction of urea and urease as well as reacting amino methylamine with lime has been used.

The noteworthy losses affecting the nutritional quality of ammoniated animal feeds include 10% reduction of protein quality [19], an irreversible reduction in the degree of unsaturation in lipids [20], a significant drip in lysine and methionine content [21,22]. The presence of residual toxicity arising from hydrolyzed products [23] along with the potential for covalent bonding of AFB1 to proteins [24] and the loss in nutritional quality, make the ammoniation treatment processes seem less acceptable. However, under defined conditions the treatment of grains (peanuts, cotton seed, corn) and their meals with ammonia appears to be a commercially viable approach to detoxification of aflatoxins to the extent of 99% [8] particularly for feed purposes.

Ammonia degradation proceeds through hydrolysis of lactone ring, and is followed by decarboxylation to produce non-toxic compounds. Due to severity of aflatoxins contamination in selected agricultural commodities, in various locales, specific decontamination processes have been approved and put into use [8,25]. Ammoniation of feeds is authorized by Food and Drug Administration of the U.S.A. In the U.S.A., Arizona, California and Texas permit the ammoniation of cotton seed products, and Texas, North Carolina, Georgia and Alabama have approved the use in aflatoxin-contaminated corn. Mexico and South Africa have approved the procedure for use on corn. Treated peanut meal is widely used in animal feeds in Europe and elsewhere; consequently the process is routinely used in France, Senegal, Sudan and Brazil. Several member countries of the European Community import ammonia treated peanut meal on a regular basis. Ammonia treatment processes for feed mill and at farm level have been worked out intensively by Park *et al.* [25]. Low ammonia concentration (0.2 to 2.0%) at high pressure (35-50 psi) and high temperature (80-120ºC would require less time (20-60 minutes) as compared to high ammonia concentration (1-5%) at atmospheric pressure, ambient conditions needed more time (14-21 days) for treating feed materials containing 12-16% moisture [25] at feed mill level and form level, respectively. Shannaz and Ghaffar [26] studied the use of ammonia gas in the reduction of aflatoxin and aflatoxin producing fungi in sunflower seeds. Use of ammonia gas reduced the seed germination but infection of *A. flavus* decreased with consequent reduction in aflatoxin production. Feeding lactating cows with ammoniated peanut meal can result in reduced levels of AFM1 in milk of cows [27]. Namazi *et al.* [28] demonstrated that 0.9-1.0% ammonia inhibited fungal growth together with aflatoxin production.

Pathological and histo-pathological examinations made with experimental and farm animals fed with ammoniated meals did not show any signs of aflatoxicosis. Also there were no differences in egg production and immunological responses in poultry. Sodium bisulfite can react with aflatoxin B1, G1, M1 and aflatoxicol at various temperatures and concentrations at various times to form water-soluble products [29]. Potassium bisulphite is a common food preservative and does not pose any consumer resistance problem [30]. Aflatoxin containing copra at moisture contents of 24% and 7% was effectively detoxified by ammonium hydroxide (> 97% and 89% reduction, respectively) [31]. Sharma *et al.* [32] prevented aflatoxin formation in the commodities like peanut and corn samples by the treatment with an aqueous solution of 2-chloroethylphosphoric acids. Bullerman [33] studied the effects of cinnamon on growth and aflatoxin production by known toxigenic strains of *A. parasiticus*. It was observed that the cinnamon is an effective inhibitor of aflatoxin production even though mycelium growth may be permitted.

Aflatoxin production by *Aspergillus parasiticus* was markedly checked by O-vanillin on the cereals and oil seeds by Bilgrami *et al.* [34]. Maximum inhibition was recorded on rice (85.6%) followed by groundnut (76.25%), wheat (54.2%), maize (52.3%) and mustard (51.1%). O-vanillin did not have any pronounced effect on seed germination.

The prevention of aflatoxin producing fungi and aflatoxin through some known anticarcinogenic compounds viz., Redoxon (Ascorbic acid 0.1 g/ml) [35] and Serpasil (Reserpine 2.5 mg/ml) [36] at different concentrations [37]. It is evident that both these drugs had shown inhibitory effects on aflatoxin production at all concentrations though in varying degrees.

Ozone effectively degraded AFB1 and AFG1 in 4% dimethyl sulfoxide at room temperature within a few minutes. The treated products were confirmed to be non-toxic by various methods. It is reported to reduce AFB1 levels by 91% in cottonseed meal containing 22% moisture after treatment at 100ºC for 2 h; however, with peanut meal (30% moisture) the reduction was only 78% after exposure to ozone for 1 h. [38]. The destruction and detoxification of aflatoxin B1, B2, G1 and G2 (50 µg/ml in 4% dimethyl sulfoxide) with ozone were confirmed by Maeba *et al.* [39].

2. MATERIALS AND METHODS

2.1. Chemical Method

2.1.1. Decontamination/Detoxification by Taxol

Taxol is an anti cancerous drug obtained from *Taxus brevifolia*. Authentic sample of taxol (paclitaxel) was obtained from Sigma chemicals. A sample of 0.02 mg was dissolved in 1ml of 100% methanol. From this stock different concentration of taxol viz., 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µg was taken and added to 100 ml of Yeast Extract Sucrose medium (2% yeast, 20% sucrose) [40] separately. Then the medium was inoculated with a disc of *Aspergillus flavus* isolate from the surface sterilized sundried sunflower seed used for oil crushing and incubated for 8 days at 30ºC as a stationary culture.

After 8 days, the cultures were killed and the culture filtrate was filtered through Whatman's No.1 filter paper. One hundred milliliter of culture filtrate was extracted thrice with equal volume of chloroform. The chloroform extract was dried over rotary evaporator. The final residue was dissolved in 0.2 ml of chloroform. The same procedure was followed for control without adding taxol.

2.1.2. Quantification of Aflatoxin B1 by TLC

Five, 10, 20 and 40 µl of the above extracts were applied to pre-coated TLC plates (Merck) along with the standard aflatoxin B1. The plates were developed in a tank containing chloroform: acetone in the ratio of 88:12. After the development, the plates were viewed under long UV light at 365 nm. Blue-fluorescence similar to standard aflatoxin B1 indicated the presence of aflatoxin B1. Quantification of aflatoxin B1 was made by evaluating on the plate itself using long UV light. The role of taxol on detoxification was determined by quantifying the intensity of blue fluorescence of aflatoxin B1.

2.1.3. High Performance Thin Layer Chromatography

Twenty micro liter of the above sample extracts were loaded onto pre-coated silica gel plate. The plate was developed in a saturated tank containing tertiary butyl methyl ether: methanol: water in a ratio of 9.6: 0.3: 0.1. The developing distance of the plate was up to 80mm. The developed plates were scanned in a Camag TLC Scanner 3 at 366 nm. The presence of blue-fluorescence indicated the presence of aflatoxin and confirmed with authentic sample.

3. RESULTS AND DISCUSSION

An attempt has been made in the present investigation to prevent aflatoxin production using an anticancerous drug taxol. Taxol (Paclitaxel) is a well known drug for it anticancerous property. It was obtained from *Taxus brevifolia* and it was also obtained from the endophytic fungi present in *Taxus brefivolia* [1], *Pestalotiopsis terminaliae*, an endophyte of *Terminalia arjuna* [41], and *Pestalotiopsis versicolor* and *Phyllosticta murrayicola*, a pathogenic fungi [42]. It is mainly used to treat breast and ovarian cancers. Therefore, this drug is specifically selected to screen its activity on the control of *A. flavus* and AFB1 production at various concentrations.

From authentic taxol (0.02 mg) (Sigma chemicals), 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µg concentrations were selected and amended with YES medium containing *A. flavus* isolate. This isolate was used as control, its dry weight was 3.9 g and its AFB1 content was 36 ppm (**Figure 2**, **Table 1**). Except 2.5 µg concentration of taxol, all the other concentrations showed marked reduction in the mycelial dry weight and AFB1 production. But there was no correlation between the mycelial dry weight and the AFB1 production. The dry weight of the mycelium ranged from 2.6-4.6 g (**Table 1**).

Among the 6 concentrations used, 3 µg of taxol was found to be suitable to control the growth and AFB1 production (**Table 1**, **Figure 8**). The content of AFB1 found at this concentration was 6 ppm by TLC and 6.3 ppm by HPTLC. The complete elimination of AFB1 might require higher concentrations of taxol (**Figures 1-8**). This study indicate that the chemical taxol was a effective inhibitor of aflatoxin production even though mycelial growth may be permitted. Taxol is quite expensive drug and we could not take this drug as food additives but the availability of *Sargassum wightii* is inexpensive and safe [15].

The U.S. FDA has currently established action levels (max) of aflatoxin to be 20 ppb for human foods (except milk), 0.5 ppb for milk, 20 ppb for animal feeds, except some cases of feeds meant for maturing and finishing

Table 1. Quantification by TLC and HPTLC.

S.No.	Concentration of Taxol (μg)	Mycelial dry weight (g)	Level of Aflatoxin B1 (ppm)	
			Quantification by TLC	Quantification by HPTLC
1	0.5	2.9	8	9.2
2	1.0	2.7	12	11
3	1.5	2.6	16	15
4	2.0	3.6	12	12
5	2.5	4.6	12	11
6	3.0	2.9	6	6.3
Control	-	3.9	30	36

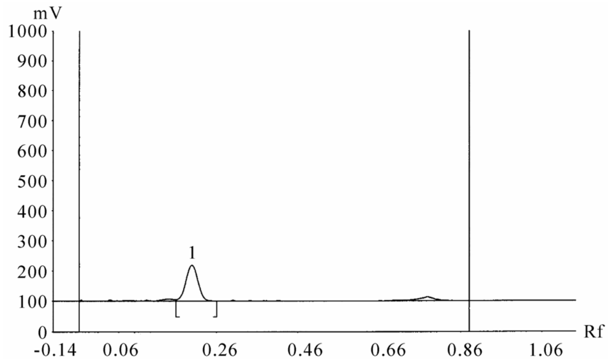

Figure 1. Authentic Aflatoxin B1.

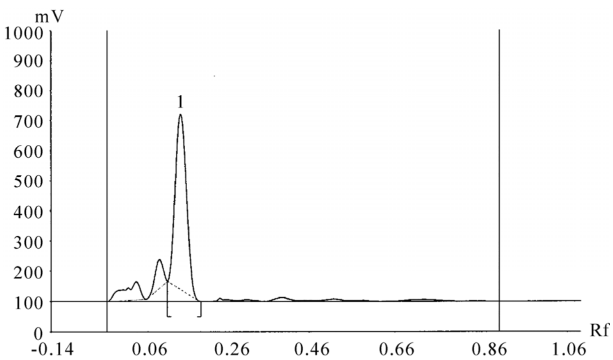

Figure 2. Control.

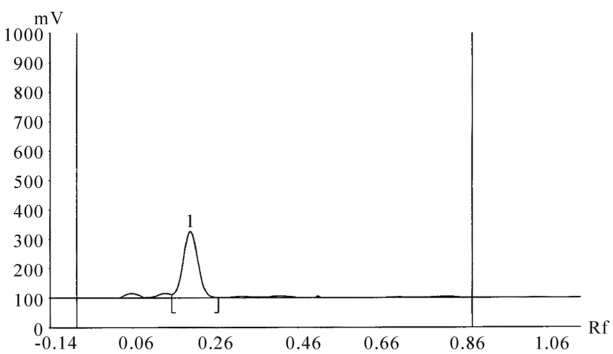

Figure 3. *Aspergillus flavus* with 0.5 μg of Taxol.

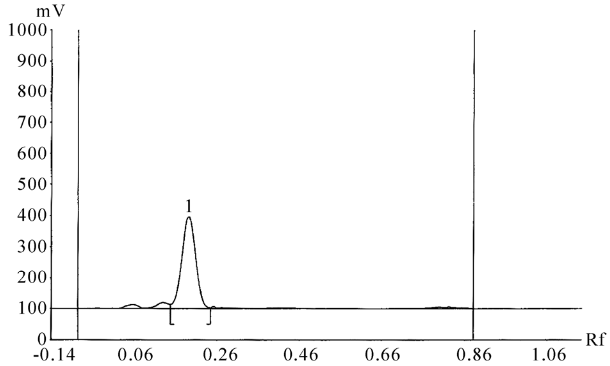

Figure 4. *Aspergillus flavus* with 1μg of Taxol.

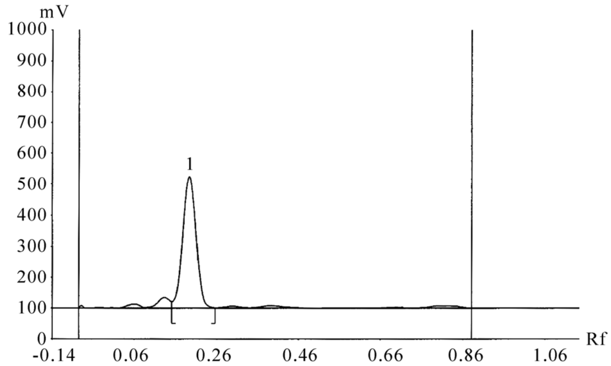

Figure 5. *Aspergillus flavus* with 1.5μg of Taxol.

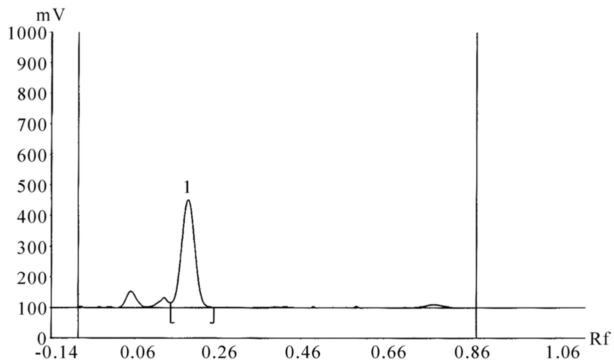

Figure 6. *Aspergillus flavus* with 2.0μg of Taxol.

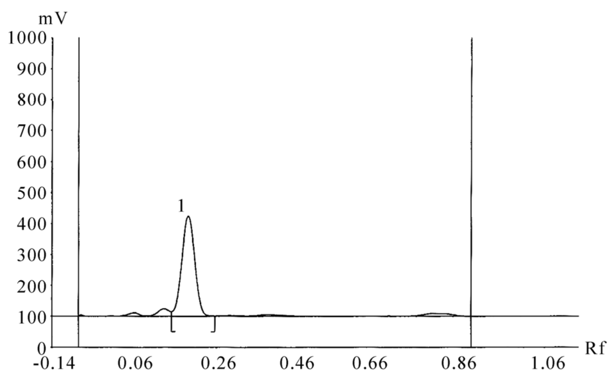

Figure 7. *Aspergillus flavus* with 2.5 μg of Taxol.

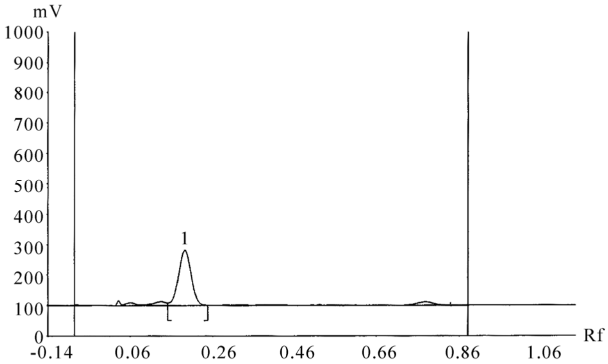

Figure 8. *Asper.gillus flavus* with 3.0 μg of Taxol.

of meat animals, which varied from 100 to 300 ppb [8].

From the viewpoint of health and economics, it is imperative that such low levels of aflatoxin are prescribed to follow up. To achieve such low levels, decontamination/ detoxification procedures are useful in order to recuperate mycotoxin contaminated commodities. The ideal decontamination procedure should be easy to use, inexpensive and should not lead to the formation of compounds that are still toxic or may reverse to reform the parent mycotoxin or alter the nutritional and palatability properties of the product. Hence approaches involving physical and biological methods have been made to detoxify aflatoxin in food and feedstuffs by many workers.

It was reported by Dollear [38] that in the case of groundnut oil, the oils were alkali refined, the soap stock removed and the oils subjected to two washing. No aflatoxin could be detected in 100 ml of the refined, washed oils. It is evident that conventional processing practices remove completely any aflatoxin that may be found in crude oils. Banu and Muthumary [43] were also found the absence of fungal spores and aflatoxin in refined oil collected from Tamil Nadu Agro Industries Corporation. Chlorophyllin (CHL) has been found to be a safe and effective agent for chemoprevention in humans exposed to aflatoxin [44]. Substitution of antifungal and aflatoxin inhibitory chemicals by natural compounds such as thyme oils is recommended [45]. Examination of various concentrations of thyme essential oils on the growth of *A. parasiticus* showed promising prospectus on the utilization of natural plant oils and extracts.

Prevention of aflatoxin elaboration has received considerable attention. Various fungicides, fumigants and chemicals [46,47], plant extracts [48], antibiotics [49] have been suggested for controlling the growth of aflatoxin producing fungi as well as aflatoxin production. Ranjan and Sinha [37] used two anticarcinogenic compound viz., Redoxon [35] and Serpasil [36] on AFB1 production and *A. flavus* control. Complete inhibition of AFB1 was noticed at higher concentration of redoxon. Combination of serpaisl and redoxon also significantly inhibited aflatoxin production as well as mycelial growth.

4. CONCLUSIONS

By this study, it is evident that the taxol had inhibitory effects on aflatoxin production at all the concentrations mentioned though in varying degrees. It is apparent from this study that the anticarcinogenic drug taxol can also be exploited for the prevention of aflatoxin production by *A. flavus.*

5. ACKNOWLEDGEMENTS

We greatly acknowledge the Department of Science and Technology, Govt. of India for providing the financial support to carryout the research work.

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Vol.2, No.7, 796-803 (2010)
doi:10.4236/health.2010.27120

Health

HIV clinic caregivers' spiritual and religious attitudes and behaviors

Elizabeth A. Catlin[1]*, Jeanne H. Guillemin[2], Julie M. Freedman[3], Mary Martha Thiel[4], Sandra McLaughlin[5], Cheryl D. Stults[6], Marvin L. Wang[7]

[1] Ethics, MassGeneral Hospital for Children, Department of Pediatrics, Harvard Medical School, Boston, USA; *Corresponding Author: ecatlin@partners.org
[2]Sociology Department, Boston College, Boston, USA
[3]Infectious Disease Unit, Massachusetts General Hospital, Cambridge, USA
[4]Clinical Pastoral Education, Hebrew Senior Life/Hebrew Rehabilitation Center, Boston, USA
[5]Department of Social Services, Massachusetts General Hospital, Cambridge, USA
[6]Sociology Department, Brandeis University, Waltham, USA
[7]MassGeneral Hospital for Children, Massachusetts General Hospital, Boston, USA

Received 10 March 2010; revised 15 March 2010; accepted 16 March 2010.

ABSTRACT

Based on prior research, we hypothesized that staff in an outpatient clinic caring for an HIV patient population might rely on religious and spiritual frameworks to cope with the strains of their work and that their responses to a spiritual and religious survey might reflect work-related spiritual distress. Surveys were completed by 78.7% of staff (n = 59). All respondents scored in the "moderate" range for religious and spiritual well-being as well as existential satisfaction with living. The large majority agreed that the religious and spiritual concerns of patients have a place in patient care. Nurses, (88.2% of nurse respondents) viewed assessing the spiritual needs of patients as their responsibility, (p = 0.03). While 82% of HIV clinic respondents privately prayed for patients always, often or sometimes, this did not include physicians. Physicians in this clinic setting appeared to be less spiritual and religious, based on their survey responses, than coworkers and than US physicians in general. The majority of clinic physicians (78%) believed that God does not suffer with the suffering patients, in contrast to the majority of support staff (69%) and nearly half of the nurses, who believed that God does suffer with them, (p = 0.018). Contrary to our expectation, respondents did not report work-related spiritual distress, which may be related to improved therapies that can prolong and improve patients' lives. Survey data revealed, however, a surprising level of engagement in and reliance on spiritual and religious frameworks among nurses and support staff. Whether the absence of measured spiritual distress is linked, in a causal rather than random manner, to spiritual and religious reliance by certain of these health care providers, is unknown.

Keywords: Spirituality; Religion; Caregivers; Caregiver Burden

1. INTRODUCTION

The emergence of AIDS in the early 1980's [1,2] presented formidable challenges to healthcare providers and hospitals. Mortality and morbidity caused by infectious diseases had progressively declined in the twentieth century; better sanitation, childhood vaccination, and the introduction of antibiotics dramatically reduced the risk of serious epidemics. Instead, hospital staffs grew familiar with mortality from cancer, heart disease, stroke, and other conditions associated with longer life spans and industrial societies. The organizational response of hospitals to the spread of HIV disease was the formation of specialized infectious disease units. In the subsequent nearly thirty years, advances in technology have substantially prolonged and improved the quality of life for treated individuals with HIV disease, increasingly identified as a chronic condition. Consequently, the medical management of patients with HIV disease resembles that of other life-threatening conditions: the trajectory of a patient's life can be extended by reliance on technology and clinical care, if available.

The epidemic, while fundamentally different, continues [1]. Worldwide, 0.8% of adults are estimated to be infected with HIV; resource-limited regions bearing the greatest disease burden [3]. The estimated number of persons in the U.S. living with HIV disease is 1.2 million, 35,962 have progressed to AIDS [4]. Hospital staffs remain responsible for chronic ambulatory care of these individuals as well as for difficult, end-of-life care [5]. In addition, attention has turned to the suffering, physical and non-physical, of patients and to the potential role of spirituality in the clinical setting. In 2006, for example, the U.S. Department of State issued guidelines for global AIDS relief that included spiritual care "that addresses the major life events that cause people to question themselves, their purpose and their meaning in life" [6].

In this article we pose the question of whether hospital staffs that care for patients with HIV disease have a sense of spiritual or religious dimensions in their work. In a previous article [7] this and related questions were asked of those who work in the newborn intensive care unit (NICU) context, where caring for critically ill and dying newborn babies raises existential and spiritual concerns. These physicians, nurses, and other providers, as they met their NICU responsibilities, were privately concerned with the meaning of suffering and death and were also aware of the need for pastoral support. In the present study, we test the hypothesis that HIV clinic providers might rely on spiritual and religious frameworks in caring for their patients and that work-related spiritual distress might be a common theme for them.

2. METHOD

This 65-item questionnaire study was approved by the Human Studies Subcommittee of the Institutional Committee on Research, [Supplement]. The instrument used was a version of the aforementioned NICU questionnaire. It was revised for care providers of adult patients, with added spiritual well-being questions [8]. From March through July 2003, the survey was made available on line to all staff members of an ambulatory HIV clinic in a large metropolitan hospital, at a workstation away from the immediate patient care area. Respondents were also given the opportunity to write in comments, for example, to questions concerning the particular difficulties of their work. Participation was voluntary and anonymous and a gift coupon was provided as reimbursement upon survey completion. A Microsoft Access 97 relational database was used to obtain and store responses from the questionnaire-assigned data table.

We surveyed 59 of the 75 staff members (78.7%). The respondents included 23 physicians, 17 nurses, and 19 administrative and support staff. The complete data set of survey responses was entered into a Microsoft Excel spreadsheet and analyzed using the statistical software program SPSS version 13.0. Frequency distributions were produced, cross tabulations performed, and Pearson's Chi-square statistics calculated on the independent demographic variables of position, age, years worked in the HIV clinic, years worked in the hospital, race/ethnicity, education, religion, marital status, parental status, and gender against other relevant variables. Significance was assigned for p values less than 0.05.

3. RESULTS

3.1. Staff Characteristics

Most participants (49.2%) were 30-39 years of age, followed by 20.3% in the 40-49 years' group, 16.9% in the 20-29 years'group, and 13.6% were 50 years of age or older. There were more female respondents (34, 57.6%) than male (25, 42.4%), (**Table 1**). The support and administrative staff were predominantly female (16/19), and males comprised 70% of the physician staff. The majority of respondents reported their race as white; less than 10% identified themselves as African-American or Asian (**Table 2**). One third (35.6%) of the HIV clinic staff reported being parents; 49.2% of the staff were married or partnered.

3.2. Religious Identification and Spiritual Assessment

The largest portion of staff, 28.8%, identified themselves as Catholic, 18.6% as Jewish, 10.2% as Protestant, 6.8% Christian Orthodox, 6.8% selected "other", 5.1% Episcopalian, 1.7% reported being Buddhist, 1.7% Quaker, and 13.6% of staff reported no religious affiliation. However, of physician staff, one fifth (20%) answered that they had no religious affiliation. All survey respondents scored in the "moderate" range for spiritual well being (41 to 99 score); similarly, 98.3% scored in the "moderate" category for religious well-being. For satisfaction with life, 98.3% indicated moderate levels. The great majority of the staff acknowledged that spiritual or religious concerns were important in patient care. When asked if the spiritual/religious concerns of a patient have a place in patient care in your view, 49.1% answered "always", "often" or "yes" (2 responses), and another 44.1% (26) answered "sometimes"; 6.8% (4) answered "seldom" (see **Table 3**). Overwhelmingly, the respondents answered affirmatively, 93.2%, to the question of the appropriateness of attending to spiritual/religious concerns in the course of caring for patients while only 4 staff members answered "seldom" and no one replied "never".

The HIV clinic staff was asked how frequently they assessed the spiritual or religious needs of patients and 22% answered "always", "often" or "yes" and 37.3% answered "sometimes". The remaining respondents were more hesitant, 40.7% (24) answering "seldom" or "never" (see **Table 3**). When asked if they felt competent to do a basic spiritual assessment, 25.8% answered "always" or "often" while 37.9% responded "sometimes" and remaining staff (36.1%) indicated "seldom", "never" or "no". Nearly two thirds of staff across the various job categories indicated that they were ill equipped to do a basic spiritual assessment. Lack of training in spiritual assessment was cited by more than half of the staff and another 38.5% replied that they were too busy or that

Table 1. Basic demographics: complete cases.

		Frequency	Percent
Position	Physician	23	39%
	Nurse	17	28.2%
	Support and Administrative Staff	19	32.2%
	Total	59	100.0%
Age in years	20-29	10	16.9%
	30-39	29	49.2%
	40-49	12	20.3%
	50+	8	13.6%
	Total	59	100.0%
Years in HIV	< 1	6	10.2%
care	1-4	21	35.6%
	5-9	15	25.4%
	10+	15	25.5%
	missing cases	2	3.3%
	Total	59	100.0%
Years at study institution	< 1	11	18.6%
	1-4	24	40.7%
	5-9	14	24%
	10+	10	17%
	Total	59	100%
Parent	Yes	21	35.6%
	No	38	64.4%
	Total	59	100%
Gender	Female	34	57.6%
	Male	25	42.4%
	Total	59	100%

Table 2. Basic demographics with missing cases.

		frequency	percent	valid %
Race	African-American	3	5.1%	5.4%
	Asian	2	3.4%	3.6%
	White	49	83.1%	87.5%
	Other	2	3.4%	1.8%
	missing cases	3	5.1%	1.8%
Marital status	Single	22	37.3%	40.0%
	Married	21	35.6%	38.2%
	Partnered	8	13.6%	14.5%
	Divorced	4	6.8%	7.3%
	missing cases	4	6.8%	
Religion	Jewish	11	18.6%	20.0%
	Catholic	17	28.8%	30.9%
	Episcopalian	3	5.1%	5.5%
	Christian Orthodox	4	6.8%	7.3%
	Quaker	1	1.7%	1.8%
	Protestant	6	10.2%	10.9%
	Buddhist	1	1.7%	1.8%
	None	8	13.6%	14.5%
	Other	4	6.8%	7.3%
	missing cases	4	6.8%	
Education	High School/GED	3	5.1%	5.3%
	Associates Degree	4	6.8%	7.0%
	Bachelors Degree	10	16.9%	17.5%
	Masters Degree	16	27.1%	21.8%
	MD	25	39%	40.4%
	Other	1	1.7%	1.8%
	missing cases	2	3.4%	

Table 3. Staff self-assessment of spiritual care giving.

	Spiritual concerns have role in patient care	I assess patients' spiritual/ religious needs	I am competent to perform a spiritual assessment*
Always or Often	**49.1%**	**22%**	**25.8%**
Sometimes	**44.1%**	**37.3%**	**37.9%**
Seldom or Never	**6.8%**	**40.7%**	**36.1%**

*(1) case missing

spiritual assessment was not part of their job description.

When asked to identify which members of the staff should be given the responsibility for assessing a patient's spiritual needs, most respondents chose social workers (88.1%), nurses (62.7%), attending physicians (61%), followed by medical fellows (57.6%). Nurses, in particular (88.2% of nurse respondents), viewed assessing the spiritual needs of patients as their responsibility, ($p = 0.03$), in keeping with the long-term historical integration of spiritual care into the discipline of nursing. There was not a chaplain assigned at this time to the HIV clinic team; staff responses to this and related questions may have been influenced by this fact.

When asked which members of the team should include consideration of the patient's religious/spiritual needs in planning their care, 93.2% selected the social worker, 79.7% indicated the attending physician and 71.2% indicated the fellow. Physicians and nurses in particular favored the fellow's taking this responsibility, ($p = 0.016$). Fewer respondents (42.4%) indicated the intern or resident, while 76.3% indicated the nurse and 71.2% the nurse practitioner or physician's assistant. Note that in this question, respondents were asked to select as many individuals as they thought should be involved in this process.

Hospital chaplains were favored by 40.7% of staff when asked what resources they drew on to help respond to a patient's spiritual needs. A patient's clergy person was selected by 32.2%, colleagues by 42.4%, while 18.6% indicated that they would turn to reading or research. 22.0% of staff replied that responding to a patient's spiritual needs is not within the scope of their practice: eight of these respondents were support and administrative staff ($p = 0.03$).

3.3. Staff Experience

The staff experience in HIV work varied but overall was modestly long-term. Ten of the 12 attending physicians were experienced in HIV work for five or more years. Twelve of the 17 nurses were experienced for five or more years. The same was true for the three social workers and for 4 of the 16 support and administrative staff.

Concerning stress relief, many chose physical exercise (61%), watching movies or reading (44.1%) and a few indicated they relied on alcohol (3) or drugs (2). About one third of the respondents (30.5%) indicated they relied on "personal spiritual practice". More indicated that they participated in social activities (50.8%) or relied on family and friends (54.2%). Some respondents sought psychotherapy (15.9%) for relief of stress.

When asked, "Do you think spiritual caring is an appropriate part of your caregiving role?" 72.9% of the HIV clinic staff surveyed responded affirmatively. Physician staff differed somewhat in this response category, as nearly 40% felt that spiritual caregiving was not an appropriate part of their role in the clinic. Of the clinic staff, 30% indicated that they always or often privately prayed for their patients, but most respondents (66.7%), when asked if they personally prayed with patients when they were with them answered, "never", (**Figure 1**, **Table 4**).

When asked if they would like to offer to pray with patients, (22) responded "never". These included ten of the 12 physicians (all six attending physicians, three of the five fellows, and the one intern) who had responded that spiritual care giving *is* an appropriate part of their professional role. Others responded "sometimes" (35.9%), and very few (3) indicated "always", "often", or just "yes", (**Table 4**). Despite this reluctance, most staff indicated a willingness to respond to a patient's request for prayer. Asked to respond to, "If a patient asks for prayers I get someone else to do it who is capable", the staff members who valued spiritual caregiving were divided into approximately one-third who answered negatively and two-thirds who responded affirmatively. Most of the latter indicated that they would get someone else "sometimes", while (8) indicated "always" or "often". When asked to respond to, "If a patient asks for prayers I don't follow through on their request", 78.1% of those responding to the question answered "seldom" or "never" (see **Table 5**).

3.4. Hardest Part of the Work

Respondents were asked to define the hardest part of their work in the HIV clinic. The responses fell into three general categories. One concerned technical and informational obstacles, that is, not having a cure or not being able to keep up with all the available and relevant information. The second category of responses concerned personnel and bureaucratic difficulties, such as difficult colleagues or tedious paperwork. The third category included respondents that cited suffering for and anxiety about patients as the hardest part of their work. Of the 49 who responded to this survey question, physicians tended to be more concerned about technical obstacles, while nurses responded that personnel/bureaucratic issues were the hardest part of their work (p = 0.002). Compassion for the patients in answering this question was widely evident but support staff displayed the most concern.

Examples of the open text responses to, "What is the hardest part of your job?" included:

"Dealing with the inefficient and wasteful delivery of care to our patients which leads to fewer resources available for all."

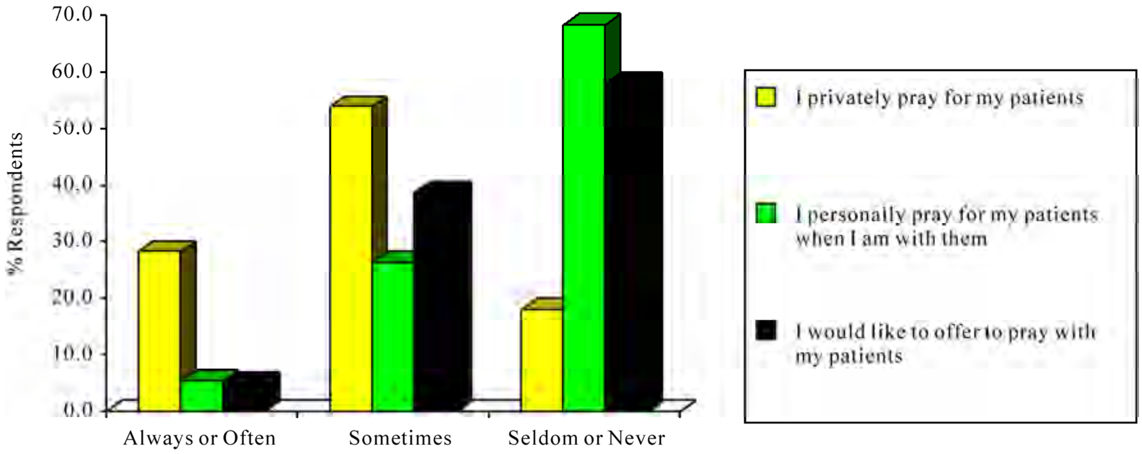

Figure 1. Prayer practices reported by clinic staff.

Table 4. Prayer practices reported by clinic staff.

	I personally pray for patients when I am with them (n = 39)	**I would like to offer to pray with patients (n = 39)**	**I privately pray for my patients (n = 40)**
	frequency	frequency	frequency
Always or Often	**3**	**3**	**12**
Sometimes	**10**	**14**	**21**
Seldom or Never	**26** 11 *of* 12 *MDs replied "NEVER"**	**22** 10 *of* 12 *MDs replied "NEVER"**	**7** 2 *MDs replied "NEVER"**

*Each of these physician respondents had replied that spiritual care giving is an appropriate part of their professional role.

Table 5. Spiritual caring as an appropriate part of role: when a patient asks for prayers.

	I do it myself	**I get someone else to do it**	**I don't follow through on their request**
	frequency (**n = 39**)	frequency (**n = 43**)	frequency (**n = 32**)
Affirmative (Always, often, sometimes)	**30**	**26**	**7**
Negative (Seldom or never)	**9**	**17**	**25**

"Telling someone they are HIV positive."

"Watching the patients suffer with this terrible disease."

"Being unable to alleviate or attenuate someone's suffering, or unable to help a person find hope or peace in their life."

3.5. Configuring the Spiritual Meaning of Suffering

When asked what theological sense or ultimate meaning they made of the suffering of patients, none of the staff indicated a belief that the patients were being punished, whether for original sin, or the sins of a partner. Some saw causality based in ignorance regarding risk for contracting HIV (10). Some also selected the reply, "We live in an imperfect, fallen world" (12). The theological sense made of patient suffering was thought by only one respondent to be due to God wanting to teach a lesson; a small number (7) indicated that patients suffered because God had a plan. No clinic staff indicated that God wanted to punish patients or that, "The devil has a hold on this world". However, many respondents believed, "God suffers with them" (26) as a way of making sense of the suffering. These included five physicians, nine nurses, the three social workers and nine of the support and administrative staff. This finding was statistically significant, ($p = 0.018$), meaning that clinic position category strongly predicted the response, "God suffers with them". Specifically, nurses and support/administrative staff were more likely to respond that, "God suffers with them", while physicians were more likely to state that, "God does not suffer with them". Overall, 72.9% of respondents did not feel that there were other explanations for patient suffering; the remaining 27.1% felt the explanation was not listed. Those that believed other reasons explained patient suffering wrote open text responses such as:

"The answer to this is probably not knowable."

"The world is not fallen but is imperfect."

"My role in care is to ameliorate suffering."

"Suffering allows redemption."

"We don't understand the meaning of their suffering."

Of the seven female physician staff, five felt that God did not suffer with the sufferers, whereas the female nurses were more evenly split between believing God suffered with (5) and did not suffer with the patients (6). Female support staff was more likely to feel that God suffered with the patients (11). Most male physicians (13 of 16) felt God did not suffer, while of male nurses, two-thirds felt that God did suffer with patients. Several staff (12) indicated that, "There is no meaning to their suffering", that, "God is not able to prevent such suffering" (13), or that, "God chooses not to prevent such suffering to give us free will" (15). A smaller number of respondents (9) indicated that, "There are accidents in nature" as an explanation for why patients suffer.

When asked about the suffering of the families and friends of those with HIV disease, 35.6% indicated that the families and friends suffered from the stigma associated with the disease but nearly all (96.6%) rejected the idea of a punitive God, original sin, or the devil as having any role in this problem. Some thought the suffering might be part of God's plan, that the world was imperfect, that there are accidents in nature or that God could not prevent such suffering (15). Of these 15 respondents, nurses were the least likely to believe that God could not prevent such suffering. Some respondents (14) indicated that, "God chooses not to prevent such suffering to give us free will". As with the suffering of patients, more staff (39.0%) thought that God suffers with the families and friends of patients. Physicians, at 6.8%, were the least likely to reply that God suffers with families and friends of the patients.

Asked what theological sense they made of their own suffering in caring for patients with HIV and their families/friends, only three respondents (a physician, a nurse, and a social worker) indicated that they did not suffer when caring for patients with HIV and their families or friends. All of the other respondents rejected the explanatory options of: God is punishing me, the devil has a hold on this world, or I suffer because of the social stigma of working with HIV patients. A few (6) indicated that they saw no meaning in their own suffering; (11) indicated that God cannot prevent suffering and the same percent indicated that God chooses not to prevent suffering to give us free will. The largest number of respondents (14) chose the option that, "God suffers with me".

When asked to choose a phrase that best expressed their attitude toward "your own suffering in your work", a few (7) opted for, "I personally do not experience suffering in my work". In contrast, 52 chose other options. The largest response group (49) chose the option, "I cannot change the fact of human suffering but I can alleviate it as much as possible". No one chose the option, "I cannot change the fact of human suffering and there is nothing I can do about that". The option, "I am willing to suffer to alleviate the suffering of others" was selected by (15); eleven of these were physicians, including six attending and four fellows, two were nurses, one was a social worker, and one was support and administrative staff, ($p = 0.007$). Physicians reported being more willing to suffer for their patients at 47.8% while the large majority of clinic nurses and administrative staff replied that they were not willing to suffer (88.2% and 89.5%, respectively) to alleviate the suffering of others.

4. DISCUSSION

Rapid advances in biotechnology have been associated with longer survival and improved quality of living for many patients with HIV disease. Nonetheless, it remains a profoundly life-altering illness, challenging patients, families, friends and caregivers and causing suffering and anguish. Many Americans rely on religion as a coping mechanism when they are seriously ill, including those with HIV disease [9,10] and those experiencing severe stress [11]. We speculated that staff caring for this patient population might also rely on spiritual and religious frameworks to cope with doing this work and we wondered whether their self reports of well-being might reflect distress. This new survey data provides surprising and unexpected information about both areas of interest.

Many staff members in this HIV clinic appeared invested in their spiritual lives and religious beliefs. The fact that 82% of them always, often, or sometimes prayed for their patients is telling, but this did not include physicians, whose replies tended to be more secular. Physicians in this clinic setting appeared to be less spiritual and religious, based on their survey responses, than their clinic coworkers and than American physicians in general. One in three reported having no religious affiliation, whereas U.S. survey data indicate that one in ten physicians reported having no religious affiliation [12]. Despite believing that spiritual caregiving was an appropriate part of their professional duties, these physicians often didn't pray or want to pray for their patients. How these providers conceptualized spiritual care was not addressed in this study. The majority of clinic physicians (78%) believed that God did not suffer with the suffering patients, in marked contrast to the majority of support staff (69%) and nearly half of the nurses, who believed that God suffered with them, (p = 0.018). On the other hand, many of these physicians were willing to suffer to relieve the suffering of their patients, whereas the support and nursing staffs voiced opposition to this altruistic approach. Another interpretation of physician responses might be that these responses represented complex reactions to the clinical setting of HIV disease, in "...a post-911 environment where spiritual issues of life and death have taken on a new urgency, but feelings about them are often ambivalent and difficult to articulate" [13].

That the clinic nursing staff demonstrated a strong spiritual and religious orientation may be related to the fact that a core spiritual dimension exists in all nursing care, as taught by nursing scholars, who have studied spiritual and religious components within their profession [14,15]. In addition to the spiritual orientation and care provided by the nurses, support and administrative staff in this HIV outpatient clinic emerged as an important resource for spiritual support and caregiving to patients. They eagerly responded to patient requests for prayer and were highly compassionate about the plight of the patient population. In fact, the support and administrative staff appeared to be key care providers for patients, although not specifically recognized as such.

An unexpected result of this research was that these healthcare providers and support staff consistently scored in the "moderate" range for religious and spiritual well-being, as well as existential satisfaction. In their work with patients with HIV disease, these care providers regularly face issues of mortality, the meaning of life, and theodicy. HIV clinic patients themselves may ask very difficult questions of staff; they have in common a chronic, complex, and potentially lethal illness, each with time to reflect on existential and spiritual issues [16, 17]. The relatively high spiritual well-being scores of the care providers in this study suggest that they may have successfully dealt with some of the aforementioned issues. They may have also benefited from decades of public education and technological innovation that have changed the environment for HIV treatment. Earlier reports documented job stress and fear of infection as frequent problems characteristic among healthcare workers caring for patients with HIV disease [18,19]. Distress among healthcare providers of patients with HIV disease and patients may have declined related to the success of potent therapeutics, especially HAART regimens [20]. Patients with HIV disease are living for much longer periods, with decidedly improved quality of life; these factors allow for qualitatively different relationships to be formed between staff and patients, about which much more could be learned. Compassion for patients was clearly evident in questionnaire answers and supports the conclusion that humane, personal attachments develop between HIV clinic staff and patients, families and friends over extended periods of treatment and follow up. This continuity in positive relationships may explain some of the apparent job satisfaction and lessened sense of being oppressed by the actual patient work that was measured.

The response rate of 78.8% to the survey was numerically robust, but the study was small and its findings may have been somewhat biased against secular results, in that the staff members who chose not to participate might be predicted to be unsympathetic to this subject area. A division appeared to exist between physicians who processed the difficulties of their work in a more secular manner and the strong component of spirituality and religiosity in the nursing and support staff of this HIV clinic. Whether the spiritual and religious reliance reported by these health care providers is linked, in a causal rather than random manner, to the absence of measured spiritual distress, remains to be researched.

This survey data points the way to further social science research, quantitative and qualitative, on the different clinical contexts for treating patients infected with HIV. Of particular interest is the interplay of care providers' spirituality and religious beliefs and those of patients with HIV disease, some from minority groups with strong religious traditions, [21,22]. But of greatest and global interest, clearly, would be achieving a cure for HIV infection [23], making possible a future world without HIV/AIDS [2].

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Vol.2, No.7, 804-810 (2010)
doi:10.4236/health.2010.27121

Health

Effects of supervised movie appreciation on the improvement of college students' life meaning sense

Xinqiang Wang[1,2], Dajun Zhang[1,2]*, Jinliang Wang[1,2], Hui Xu[1,2], Min Xiao[1,2]

[1]Center for Student's Mental Health Education, Southwest University, Chongqing, China;
[*]Corresponding Author: zhangdj@swu.edu.cn
[2]Research Institute of Educational Science; Southwest University, Chongqing, China

Received 27 December 2009; revised 20 March 2010; accepted 23 March 2010.

ABSTRACT

The purpose of this study was to explore the effects of supervised movie appreciation on improving the life meaning sense among college students. The intervention combined by "pre-video, post counseling" was conducted on the experimental group, while the control group received no intervention. Results have shown that the scores on the subscales of will to meaning, life purpose, life control, suffer acceptance and on the total scale have improved significantly. No gender difference was found on the intervention effect, and participants receiving intervention maintained higher level on related subscales a week later, indicating that supervised movie appreciation is an effective way to improve the life meaning sense among college students.

Keywords: College Students; Life Meaning Sense; Supervised Movie Appreciation; Suicide Prevention; Mental Health Education

1. INTRODUCTION

The life meaning theory proposed by Frankl is of great importance in the studies of life meaning. According to Frankl's proposal, many mental disorders among college students, such as depression, emptiness, and loneliness and suicide behavior are caused by a lack of life meaning, namely, a negative mental state resulted from a mere pursuit towards natural life itself while lacking goals of life spiritual values [1]. Other studies also have shown that the life meaning plays an important role in preventing mental illness, preventing suicide, achieving mental health and improving the adaptability to the changing living environment. It can continuously predict the mental status and play a unique and irreplaceable role in case of crisis and serious frustration [1-5]. Therefore, it may effectively prevent college students' mental illness and suicide and maintain a mental healthy state, by implementing active intervention for college students [6,7]. However, few empirical studies in the psychology arena have been conducted to explore the effective ways to achieve this goal.

Educators are, in recent years, increasingly focusing on the integration of multiple approaches and modes to improve teenager's mental health [8] and strive to expand the intervention scope of mental health education for students [9]. The present education arena is in urgent need of mental health education approaches characterized by implicitness, protectiveness and permeation [10]. It is universally acknowledged that movie appreciation is an intervention approach with aforesaid advantages. Supervised movie appreciation is a psychological counseling approach by which the educators guide, on a customized basis and for certain educational objective, the participants to change the cognitive or emotional factors, including their opinions and attitudes towards themselves, the others or certain matter, develop individual's positive mental quality, relieve mental stress, prevent mental illness and eliminate mental disorders through movie appreciation and comprehension. Yuan [11] believed that individuals may gain, during the movie appreciation, the internal drive that can influence human being's emotion, thought, habit and belief, driving individuals to seek a brighter and more profound life. Meanwhile, individuals' existed values and morality is imperceptibly changed by the movie. Such influence will never end but continue throughout the life after the movie appreciation. However, the idea that movie appreciation is helpful to facilitate mental health mainly comes from theoretical explanation and empirical observation heretofore and there is few convincing empirical evidence [12-14]. Therefore, in the present study, educational experiment was used to explore the effects of supervised movie appreciation on improving students' life meaning sense.

2. METHOD

2.1. Participants

A class consisting of 56 students was selected at random, at a class of their optional courses, from those in Grade 1 of a college in Chongqing, China; they had never watched the film named POSTMAN IN THE MOUNTAINS. 21 boys (37.5%) and 35 girls (62.5%) consisted of the experimental group, with an average age of 19.67 years old and a standard age deviation of 0.92 years. For the control group, there were 46 freshmen including 20 boys (43.5%) and 26 girls (56.5%), who had not watched the film either. The average age for the control group was 19.64 years old and the standard age deviation was 1.01 years. Such participants were coming from different departments of the college and majoring in different subjects.

2.2. Material

The film named POSTMAN IN THE MOUNTAINS[1], which lasted one hour and thirty minutes approximately, was produced by Xiaoxiang Film Studio and Beijing Film Studio in 1998. The movie was shown on a 120-inch projector screen in the large multi-media classroom with a capacity of 120 people.

2.3. Measures

We used, in the objective evaluation, the subscale "Meaning Searching and Assertion" in the total scale "the Life Attitude Profile (LAP)", a measurement developed by Ho on the basis of the central concepts of Frankl's logotherapy [2]. This subscale consists of 4 dimensions and 25 items, including will to meaning (9 items), life purpose (4 items), life control (7 items) and suffer acceptance (5 items). Likert-style 5-point rating style was adopted in the subscale, with higher score indicating higher level on life meaning. Specifically, the dimension of will to meaning is to investigate individual's motivation to seek the significance and purpose of the existence of self, the dimension of life purpose is to investigate whether an individual has clear and meaningful purpose of life and the individual's satisfaction with the life purpose, the dimension of life control is to investigate whether an individual can make choice at his/her discretion and how he/she will be responsible for the life, and the dimension of suffer acceptance is to investigate whether an individual has understood what the suffering means and to what degree he/she may accept the suffering. The total scale has been shown to have relatively high internal consistency reliability, retest reliability, content validity and construct validity as well as certain predictive validity since the four subscales are all able to predict the "positive mental health" in general [2]. The Cronbach's Alpha coefficient for internal consistency reliability was 0.876 in the subscale selected in this study.

The subjective evaluation of participants` life meaning sense was also provided, based on the interview on the participants after watching the film, the feedback of 12 participants selected randomly by the experimenter from the experimental group, and the information reflected by participants and recorded by the experimenter during the experiment.

2.4. Procedure and Intervention

The intervention model composed by "pre-video, post counseling" [13] was conducted on the experimental group by means of mental health education lessons for college students, while the control group received no intervention.

2.4.1. Pre-Video Introduction

The slides on the projector screen demonstrated for the experimental group the theme of this movie, "love, hope, responsibility and meaning of life", and briefly introduced some important information, including the director, actors, actresses, awards obtained in China or other countries and the sensation caused abroad. However, such introduction did not disclose any plot.

2.4.2. Pre-Test

The experimental group was asked, by the instruction that "please write down your attitude towards the life before appreciating the movie", to fill in the "Meaning Searching and Assertion" in the "the Life Attitude Profile" before the movie was on. The subjects in the control group, who attended a class having no relation with mental health education, also took part in the pre-test.

2.4.3. Watching the Movie

The experimental group was watching the movie POSTMAN IN THE MOUNTAINS.

2.4.4. Post Counseling

The experimenter first shared with the experimental group the experiences in "beauty", "love, persistence and hope", "belief", "enrichment", "beauty of simplicity and meaning of life", "responsibility and significance of human beings" thereafter, and then asked the participants to tell their experiences in their life at their discre-

[1]The movie was entitled the Best Film, the Best Director and the Best Actor of the 19th Golden Rooster Awards, the People's Choice Award at the 32nd Montréal World Film Festival, the Silver Peacock Award, a Special Jury Prize, at the 31st International Film Festival of India, the Japanese Kinema Junpo Awards, the awards at the Mainichi Film Concours and the Awards of the Japanese Academy.

tion. Eight participants did so. They spent 2 hours and 40 minutes in total on movie-watching and post counseling.

2.4.5. Post-Test

The post-test was conducted on the experimental group immediately after the post counseling and on the control group as soon as the mental health education lessons ended. The post-test for the experimental group and the control group were conducted at the same time.

2.5. Follow-Up Study

The same measuring was conducted on the experimental group one week after the supervised movie appreciation.

2.6. Processing of Experimental Data

Classify and number the pre-test and post-test of each participant's scale according to the type of groups and make statistics and analysis with SPSS13.0. Qualitative analysis was used for subjective evaluation.

3. RESULTS

3.1. Scores of Experimental Group and Control Group during Pre-Test and Post-Test of Life Meaning

Independent-samples t test was used to compare the sores between the experimental group and the control group on the pre-test. In the post-test, independent-samples t test was also conducted to compare the difference between control group and experimental group. **Table 1** shows, in respect of the subscales and the total scale of life meaning: 1) the post-test score of the experimental group is much higher than the pre-test score ($p < 0.001$), and 2) the post-test score of the control group does not change remarkably compared with its pre-test score ($p > 0.05$). During the pre-test, the experimental group and the control group scored almost the same in respect of the subscales and the total scale of the life meaning ($p >$

Table 1. Scores of experimental group and control group during pre-test and post-test of college students' life meaning.

Subscale		Pre-Test	Post-Test	t
		M ± SD	M ± SD	
Will to meaning	Experimental Group (n = 56)	37.34 ± 5.00	40.29 ± 3.81	–5.133***
	Control Group (n = 46)	37.52 ± 4.67	37.72 ± 5.23	–0.551
	t	–0.189	2.78**	
Life purpose	Experimental Group (n = 56)	14.16 ± 2.76	16.07 ± 2.47	–7.076***
	Control Group (n = 46)	14.35 ± 3.19	14.76 ± 3.27	–1.799
	t	–0.318	2.241*	
Life control	Experimental Group (n = 56)	26.80 ± 3.22	28.48 ± 2.71	–3.854***
	Control Group (n = 46)	26.30 ± 3.71	26.57 ± 3.56	–0.719
	t	0.728	3.085**	
suffer acceptance	Experimental Group (n = 56)	21.29 ± 3.25	22.55 ± 2.70	–4.139***
	Control Group (n = 46)	20.91 ± 2.99	20.76 ± 2.85	0.531
	t	0.598	3.255**	
Total Scale	Experimental Group (n = 56)	99.59 ± 11.27	107.39 ± 8.30	–6.252***
	Control Group (n = 46)	99.09 ± 11.42	99.80 ± 12.18	–1.107
	t	0.223	3.597**	

Note: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

0.05); during the post-test, the experimental group scored much higher than the control group in respect of the subscales and the total scale of the life meaning ($p < 0.05$).

3.2. Average Increase of Post-Test Scores over Pre-Test Scores of Experimental Group and Control Group

Such increase was obtained by post-test scores minus pre-test scores of experimental group and control group. The independent-samples t test was then used to verify and compare the difference between the average increases of the two groups. Results have shown that the increase of the experimental group's scores on all subscales and the total scale of life meaning is much higher than that of the control group ($p > 0.05$) (**Table 2**).

3.3. Analysis on Gender Effect during the Supervised Movie Appreciation of Experimental Group

We took all subscales and the total scale on college students' life meaning, including two dependent variable of pre-test and post-test, as the within-subjects factors and took the gender, including two value label of female and male, as the between-subjects factors. We used the repeated measures ANOVA to examine whether there was the main effect of gender during the supervised movie appreciation experiment. Results have shown that the main effects of gender were unremarkable for the four subscales, including will to meaning, life purpose, life control and suffer acceptance, and the total scale. They were respectively $F(1, 18.229) = 0.599$, $p = 0.442$; $F(1, 5.601) = 0.476$, $p = 0.493$; $F(1, 1.371) = 0.109$, $p = 0.743$; $F(1, 30.672) = 2.055$, $p = 0.158$; $F(1, 121.072) = 0.792$, $p = 0.377$. The results showed that gender had no significant effects on the scores.

3.4. Comparison between the Post-Test Score of the Experimental Group for the First Time and the Post-Test Score One Week Later

We conducted the post-test again one week following the experiment to find out whether the effect of this intervention experiment was sustainable (1 participant is absent) and compared the results with the repeatedly measured paired-samples t test used for the post-test for the first time. Then we found that there was no significant difference between the scores of the two post-tests ($p > 0.05$). This demonstrates that the intervention still have effect on students' life meaning sense one week later (**Table 3**).

3.5. Subjective Evaluation

The participants were all conscientiously watching the movie when it was on, and some participants even took the lead to applaud as the movie was over. The experimenter observed that the participants were all moved by

Table 2. Average increase of post-test scores over pre-test scores of experimental group and control group.

	Will to Meaning	Life Purpose	Life Control	Suffer acceptance	Total Scale
Average Increase of Experimental Group (n = 56)	2.95 ± 4.30	1.91 ± 2.02	1.68 ± 3.26	1.27 ± 2.29	7.80 ± 9.34
Average Increase of Control Group (n = 46)	0.20 ± 2.41	0.41 ± 1.56	0.26 ± 2.46	–0.15 ± 1.94	0.72 ± 4.40
t	3.870***	4.120***	2.434*	3.331**	4.730***

Note: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 3. Comparison between the post-test score of the experimental group for the first time and the post-test score one week later (n = 55).

	Post-Test (1st Time)	Post-Test (1 Week Later)	t
Will to Meaning	40.29 ± 3.81	39.84 ± 3.96	1.173
Life Purpose	16.07 ± 2.47	16.04 ± 2.52	0.142
Life Control	28.48 ± 2.71	27.88 ± 2.95	1.542
Suffer acceptance	22.55 ± 2.70	22.11 ± 2.66	1.761
Total Scale	107.39 ± 8.30	105.86 ± 9.29	1.750

the movie and have ever shed tears because their canthi were wet. Most participants indicated, during the post counseling and in their feedback, that the experimenter's pre-video introduction played an important role in stimulating their interest. For example, a student surnamed Hu wrote down that "It is said that this movie can 'make all men shed tears', and this makes me interested in it. I wonder what can bring so many awards for this movie and make it so attractive?" Meanwhile, the environment of the rural areas, where the story took place, was similar to some extent to the participant's living environment. For example, a participant surnamed Wen wrote down that "It seems that the story took place in my hometown". In addition, the movie is close to our life and this is also important for the movie to receive wide identification. For example, a participant surnamed Zhang wrote down that "It is so close to my life that I feel it should be a true event instead of just a story". From the perspective of father-son relationship which has strong emotional shock, the story demonstrated many issues on life meaning, including career, love, family and responsibility. All of those also concerned the college students and may better penetrate and enlighten the participants. For example, a participant surnamed Huang and another surnamed Sheng respectively wrote down that "This is the first time that I watch a movie so conscientiously that I never miss any scene. It shocked me so much that I have received a spiritual baptism" and that "The emotion revealed is moving although the plot is plain". The experimenter guided, during the post counseling, the participants to exchange their feelings on the movie based on their experiences in the past life. Other participants applauded to reinforce the effect and emotional resonance was thus caused, which deepened the participants' comprehension on the movie and consolidated the intervention effect. To sum up, the most common words repeated by the participants were "responsibility, mission, belief, hope, expectation, and value and meaning of life", no matter whether during an immediate exchange of feelings or an after-class discussion. In addition, some participants pondered and made a further process on the movie following the experiment. For example, a participant surnamed Li wrote down that "I even pondered the movie for an extremely long time" thereafter.

4. DISCUSSION

The purpose of this study is to investigate the effect of supervised movie appreciation on improving college students' life meaning sense. The results have shown that the supervised movie appreciation may remarkably improve college students' life meaning sense. This is identical to the idea that "movie appreciation is helpful to achieve mental health" [12-14] and preliminarily proves the idea by empirical evidence. In addition, we also found in this study that the gender did not cause significant impact on the experimental results and that there was no significant difference between the scores for the post-test one week after the experiment and the post-test for the first time. This indicates that the effect of this experiment is sustainable and further proves that supervised movie appreciation is an effective mean to improve the life meaning sense among college students.

Why the supervised movie appreciation may improve the life meaning sense among college students? The reasons are: 1) it is the precondition to control and remove irrelevant variables. For example, we selected the freshmen that have not watched the movie as the participants for the experimental group and the control group and effectively controlled the variables of such participants, including the gender, the grade and the age. 2) It assures successful intervention to make an integrated and feasible counseling proposal. This experiment was conducted in accordance with BU Hong's intervention model combined by "pre-video, post counseling" [13] and the results proved that the model was feasible and played an important role in counseling. It was helpful for the experimenter to set the intervention direction for participants to demonstrate the key words during pre-video introduction and make the participants understand their life meaning before the movie began; the participant would connect the plot with the intervention theme consciously or unconsciously when they were watching the movie. Meanwhile, the introduction of some basic information on the movie was helpful to stimulate the participant's interest as said by the participant surnamed Hu during the subjective evaluation. In addition, it was also helpful to consolidate the effect when individuals' attitudes formed during the intervention can be transferred to reality situations [15]. The experimenter directed the participants during the post-counseling to talk about their feelings based on their experiences and this was helpful for the participants to further comprehend, transfer and consolidate the theme of the intervention. 3) It is crucial for successful intervention to select appropriate movie. Supervised movie appreciation is helpful to achieve students' mental health, but it doesn't mean that this objective can be realized by selecting a movie at random. The key is to find an appropriate movie that can cause strong resonance [14]. Therefore, the selection of movie is extremely important and it should follow certain principle [12]. In this study we searched a movie with a theme close to the life meaning from the Internet and from various other channels before the intervention, analyzed the basic information, introduction and review of the related movies and watched and discussed the movie before making determination. Finally, we selected POSTMAN IN THE MOUNTAINS as the intervention material to improve the life meaning sense. The cultural settings of this movie is similar to the participants' living environment to some extent, the plot seems like a true story, the picture is beautiful and the music is pleasant, so it was highly accepted. The movie stimulated much of the participants' emotion and caused great shock to them via the father-son relationship. The father and the son in the movie were faced with many issues in respect of life meaning, including career, love, family and responsibility, and such issues were just those concerning most college students and frequently discussed by them. It was not only consistent with the college students' psychological characteristics, but also close to the theme of the intervention; it may better permeate the intervention and enlighten the participants. 4) It is of significant importance whether the participant identified, catharsised and insighted the theme of the intervention during the supervised movie appreciation. How can the supervised movie appreciation improve the participant's mental quality? We believe that the functions of supervised movie appreciation may be explained from psychological "identification, catharsis and insight" [10,16]: the participants consciously or unconsciously, when watching the movie, regarded the characteristics of the protagonist as their own characteristics and deemed the roles in the movie as themselves to achieve identification; then they further experienced the emotional conflict and strain in the circumstances in the movie after they entered into the spirit of the character so that the self suppressed at ordinary times and their overload inhibition can be released and the emotion can be catharsised; finally, they gradually sublimated, insighted and internalized the theme of the intervention at the time of identification and catharsis under the direction of the movie, and the post-counseling and their preparation of feedback may further help them comprehend, internalize, transfer and consolidate the experimental effect.

There are still limitations in this study although it has been proved that supervised movie appreciation may effectively improve the life meaning sense among college students in the experimental group. 1) The scope for selecting participants is limited. Only a small number of freshmen were appropriate to this study. It has been proved via certain study [17] that the freshman's mental quality level is quite different from that of the college students in higher grade; therefore it needs further research and verification whether this experiment is effective to all college students. In addition, most participants receiving the intervention in this study were from rural areas and their living environment was quite similar to that in the movie, which may help such participants identify the movie and enhance the intervention effect. Therefore, it also needs further research whether this experiment is effective to the college students from urban areas. 2) Other subjective items, which were not related to the life meaning sense, should be designed as a control variable and included in the further research to make the result more reliable. 3) It also needs further research and investigation whether this experiment is effective no matter whether there is direction or not during the movie appreciation and whether such effects are the same or different.

5. CONCLUSIONS

The scores on the subscales of will to meaning, life purpose, life control, suffer acceptance and on the total scale have improved significantly and such improvement is sustainable to some extent. No gender difference was found on the experimental effect. Results have shown that supervised movie appreciation is an effective mean to improve the life meaning sense among college students.

6. ACKNOWLEDGEMENTS

This study was supported by the fund of School Social Work Safeguard System for College and Middle School Student's Mental Health (06XSH012), and supported by Key Project "Strategies on Cultivating College and Middle School Students' Personality Traits" at Key Humanity Social Science Research Institute in Chongqing.

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Vol.2, No.7, 811-818 (2010)
doi:10.4236/health.2010.27122

Health

Working with adolescents with mental disorders: the efficacy of a multiprofessional intervention

Gatta Michela[1]*, Pertile Riccardo[2], Testa Costantino Paolo[3], Tomadini Paola[1], Perakis Ecaterini[4], Battistella Pier Antonio[3]

[1]Pediatrics Department, University of Padua, Padua, Italy; *Corresponding Author: michela.gatta@unipd.it
[2]Department of Medicine and Public Health, Section of Psychiatry and Clinical Psychology, University of Verona, Verona, Italy
[3]Neuropsychiatric Unit for Children and Adolescents, ULSS 16, Padua, Italy
[4]Neuropsychiatric Unit for Children and Adolescents, ULSS 9, Treviso, Italy

Received 4 March 2010; revised 9 March 2010; accepted 15 March 2010.

ABSTRACT

The aim of this work was to compare multiprofessional and uniprofessional interventions applied to adolescent patients affected by psychiatric disorders. The initial hypothesis is that a multiprofessional intervention is more efficacy than a single one. A hundred individuals, 66 males and 34 females, aged between 12 and 19 years affected by emotional and behavioural problems, were selected and divided into 5 groups under the therapeutic treatment. Subjects, after diagnosis (ICD 10) and therapeutic suggestion, were clinically followed for 12 months. The Global Assessment Functioning Scale (GAF) was used to evaluate therapeutic efficacy of interventions. The outcome is associated with the type of intervention: who got clinically better are those patients who underwent multiprofessional integrated therapy rather then a single intervention.

Keywords: Adolescence; Psychopathology; Interprofessional Intervention; Compliance; Outcome

1. INTRODUCTION

The approaches to problems concerning mental health can historically be grouped into three theoretical-methodological systems: the psychological one, the bio-pharmacological one and the socio-environmental one. The operators have often the tendency to ideologically support one the above approaches thus emphasizing a dichotomy which originates from an old separation between body and mind and between individual and setting. According to this trend the therapist with biological education often tends to reduce everything to choosing the appropriate drug to eliminate the symptom. The therapist with psychological education is only interested in giving the patient the most suitable interpretation for putting him in the condition to overcome the symptom; while the educational therapist tends to search for the breakdowns of social nature which are considered the cause of the pathological behaviors, in order to suggest more adequate relational models. Everyone penned in his own shell often mistrusts the other approach running the risk of misunderstanding the patient's needs and of carrying out partial or ineffective interventions.

Many studies scrutinized and compared the benefits of distinct treatment settings for different psychopathologies (psychosis, eating disorder, mood disorder, behavioural problems, ADHD, etc) and reviewed the different treatment modalities that have proven helpful in the management of young patients [1-5]. Although different treatment settings, a multi-modal treatment approach comprising individual psychotherapy, pharmacology and family-based interventions are emphasised and recommended, nevertheless, evidence-based findings on the effect of different treatment methods are limited [6-9].

2. AIM

This work evaluates different ways of approaching adolescents with mental disorders. Its aim is that of comparing multiprofessional interventions with interventions based on a single approach and analyzing their therapeutic efficacy. The initial hypothesis is that a multiprofessional intervention is more efficient than an intervention based on a single approach. Moreover the authors want to study the influence that linkage variables such as psychiatric diagnosis, timing of intervention, therapeutic compliance, patients' participation, have with different types of treatments (educational, psychological and psychiatric ones) and different associations of treatments.

3. METHODOLOGY AND SAMPLE

The Neuropsychiatric Unit the patients were referred to, is a second level service which treats medium-severe psychopathological disorders. The structure provides for services characterized by different types of interventions (psychodynamic oriented psychotherapy, educational treatment, pharmacological therapy) chosen according both to the judgment of the specialist who visit the patient and to the service's vacancy at that moment. To verify the efficacy of different treatments, it has run a retrospective study which has analysed those treated patients for whom one year follow up was available. Patients consecutively undergone one of the three treatments (single or in association) during a six months period were 112 individuals. The period is not very long to limit the unhomogeneity of the sample. One year follow up was available for 100 out of 112 subjects.

The psychiatric disorders of the subjects were diagnosed according to ICD 10 [10]. After diagnosis, patients were suggested to undergo therapeutic treatment, on the basis of the understanding and written consent of each subject and his/her parents. Three kinds of treatment were applied: psychological one (psychotherapy with interviews once a week or twice a month, with psychodynamic orientation), psychiatric one (pharmacotherapy antidepressant or atypical antipsychotic drugs and clinical monitoring with one to three psychiatric visits every three months) and educational one (various activities such as theatre/expressive-painting/motility in team and manual laboratories, mediated by educational operators, where the patient can experience his/her abilities and limits individually or in group). These interventions were adopted singularly or in association (two or three of them). Patients were tested before and after the treatment. The Global Assessment Functioning Scale (GAF) [11,12] and Youth Self Report (YSR 11-18) [13-15] were used to evaluate therapeutic efficacy of interventions 12 months after the beginning. With respect to the GAF scale, patients were considered clinically improved, worsened or unvaried depending on the scoring reported during the retest (a difference in score of at least 10 points was required to define improvement or aggravation, otherwise the patient was considered unvaried). Clinical evolution was statistically studied in relation to other variables: psychiatric diagnosis, timing of intervention, therapeutic compliance, patients' participation, type of intervention.

Psychiatric diagnosis was formulated according to ICD 10 [10] which is the manual of mental health disorders used by clinicians of the Neuropsichiatric Unit of Padua. Timing of intervention considered a period time less then three months, a period time between three and nine months and a period time longer then nine months. Therapeutic compliance was divided and named in 'adequate' (when the patient started therapy and maintain it in accordance with the therapist), 'discontinuous' (when the patient was partially compliant, missing at least two sessions consecutively, at least once every three months) and 'with interruption' (when the patient dropped out precociously or did not follow the therapeutic indications at all). Patient's participation was evaluated on the basis of patient-therapist interaction verified throughout the analysis of clinical files, reports and minutes of sessions, interviews and equips: the WAI-O (Working Alliance Inventory-Observer version) translated into Italian language [16-18] was used. WAI-O ratings for each patient were assigned by an external clinician requested to read and scrutinize adolescents' medical records. Ratings, ranging from a minimum of 120 to a maximum of 168, were split into three groups: 120-132 (which we named as 'opposition'), 133-145 (which we named as 'ambivalent participation'), 146-157 (which we named as 'passive participation') and 158-168 (which we named as 'active participation'). Rating was carried out by a neutral observer during the clinical interviews. The type of intervention was described on the basis of the single treatment or the association of different treatments as explained in the sample section.

Data about patients were collected in an anamnesis schedule, then transferred into a computerised database for computation, which is performed using SSPS version 10 and SAS® package, rel. 9.1.3.

Statistic Analysis: descriptive analysis, performed with SSPS version 10, first included the frequencies distribution of the main variables collected in the study; then since variables were all expressed in a nominal scale, a Chi-squared test was carried out to identify the relationships between therapeutic efficacy and other variables referred to patients. A paired t-test was performed to investigate the differences in YSR's scores before and after the intervention. Multivariate analysis, performed with SAS® package, rel. 9.1.3, consisted in a multivariate logistic regression to identify those variables related to the therapeutic efficacy, while holding the other variables constant in the model. The value of $p < 0.05$ was considered significant.

Among 112 adolescents who were referred in six months time, 100 individuals had been clinically followed for 12 months. They were 66 males and 34 females, aged between 12 and 19 years, affected by emotional and behavioural problems. Their clinical files provided retrospective information about diagnostic and therapeutic processes. The sample finally taken into consideration was retrospectively divided into 5 groups under therapeutic treatment:

1) psychological treatment
2) educational treatment
3) psychological and educational integrated treatment
4) psychiatric (pharmacological) and psychological integrated treatment
5) psychological, psychiatric and educational integrated treatment

4. RESULTS

Results about frequencies analysis of variables are represented in **Tables 1-5**.

Multivariate analysis (carried out with the SAS® package, rel. 9.1.3) was performed using a stepwise logistic regression analysis (significance level for entering = 0.15 and significance level for removing = 0.10) to identify variables related to the therapeutic efficacy, which is the dependent dichotomous variable of the study.

Results of Logistic Regression Analysis for patients with an efficient therapy result (cases) compared to patients with a not efficient treatment (controls) are represented in **Table 4**.

Patients with an adequate therapeutic compliance have a probability 5,762 times higher to present a clinical improvement (p-value = 0.0076) compared with patients

Table 1. Observed distribution (frequencies and percentages) by age inter vals and sex.

		Freq	%
Age intervals	12-14 yrs	43	43
	15-17 yrs	46	46
	18-19 yrs	11	11
	Total	100	100
Sex	Male	66	66
	Female	34	34
	Total	100	100

Age categories by sex

	Male	Female	Total
12-14 yrs	32 (74.4)	11 (25.6)	43 (100.0)
15-17 yrs	28 (60.9)	18 (39.1)	46 (100.0)
18-19 yrs	6 (54.5)	5 (45.5)	11 (100.0)

Table 2. Observed distribution (frequencies and percentages) by diagnosis ICD 10 and type of treatment.

	Freq	%
Diagnosis ICD 10		
Psychotic Disorders	18	18
Affective Syndroms	21	21
Neurotic Syndroms	10	10
Personality Disorders	21	21
Soft Mental Retardation	6	6
Behaviour/emotional Disorders	9	9
Eating disorders	4	4
Comorbility (personality dis. + anxiety or mood dis.)	11	11
Total	100	100
Type of treatment		
Educational Treatment	16	16
Psychological Treatment	15	15
Educational + Psychological Treatment	19	19
Psychological + Psychiatric Treatment	15	15
Educational + Psychiatric Treatment	7	7
Educational + Psychiatric + Psychological Treatment	28	28
Total	100	100

who are not compliant. A multiprofessional intervention (p-value = 0.0242) and an active participation of the patient during the treatment (p-value = 0,014) is associated with a probability more than four times higher to obtain a clinical improvement. The last variable entered in the model is 'timing of therapy' (p-value = 0.0163): patients whose therapy lasts less than 3 months present a very lower probability (OR = 0.062, CI = 0.009-0.439) to get clinically better compared with patients whose intervention lasts more than 9 months.

The p-value of likelihood ratio test < 0.0001 indicates the efficiency of the final model. The percentages of sensitivity and the specificity are respectively 86.2% and 68.3%.

To analyse the differences in Achenbach's scores (means) before and after the intervention a paired t-test was used. **Table 5** shows there was a statistically signifi-

Table 3. observed distribution (frequencies and percentages) by timing of intervention, patient's participation, therapy eutic Compliance and clinical outcome (GAF).

	Freq	%
Timing of intervention		
< 3 months	19	19
3-9 months	41	41
> 9 months	40	40
Total	100	100
Patient's participation		
Active	53	53
Passive	17	17
Ambivalent	22	22
Total	100	100
Therapeutic compliance		
Adequate	70	70
Discontinuous	20	20
Early interruption	10	10
Total	100	100
Clinical outcome (GAF)	Freq	%
Improved	58	58
Unchanged	31	31
Got worse	11	11
Total	100	100

cant change (p-value < 0.05) in patients' Achenbach's mean scores, most probably due to the treatment efficacy. It is evident, looking at the percentages, that normal scores increased, whereas pathological ones significantly decreased.

Using the chi square test a statistically significant result about the relation between type of association of treatments and clinical evolution was obtained: the patients who got clinically better are those who underwent multiprofessional integrated therapy and in particular association between psychological and educational intervention, (63%), psychological and psychiatric intervention (71%) and the three types together (79%).

5. DISCUSSION

The sample is formed by individuals, prevalently boys (66%), basically aged between 12 to 17 years (**Table 1**). According to literature which shows as in the child-juvenile sectors of psychiatry, boys outnumber girls until the age of 12-14 years and then girls become the majority [19], in our sample too the gap between genders decreases with age, so that after 17 years of age the percentage of males and females tends to become similar (**Table 1**). **Table 2** shows the diagnosis according to ICD 10 formulated at the end of the psychodiagnostic process. The percentages are quite similar to those of literature about epidemiology of psychiatric disorders in clinic populations of adolescents and young adults [20-22]. The relevant percentage of severe psychopathology as psychosis and personality disorder must be read also within the typology of our service: a second level one which in Italy means a structure functioning in between a outpatients' and inpatients' service, where severe psychiatric diseases are recovered and treated. **Tables 2** shows types of intervention and treatments' association: the prevalence of multiprofessional interventions (association of three different therapies in 28% of cases), besides being indicative again of the complexity of patients' psychopathology, is linked to the general methodology of our Service where an approach to the adolescent that integrates different therapeutic efforts (psychiatric, psychological and educational ones) is preferred when possible to apply. About the timing of interventions, **Table 3** shows that most therapies last more than three months (81%, and 40% more than nine months). Those patients who underwent therapy for less than three months usually are patients that dropped out. Actually, being the Service one which receives individuals affected by serious psycho-pathology, suggested therapies habitually last 3 months at least. In **Table 3** it is also shown patient's participation mode in the therapeutic process: active, passive, oppositional or ambivalent. Patient's way to participate during sessions and activities is significant of the relationship with the therapist and working on the therapeutic alliance is very important for a good compliance and a positive outcome [23-25]. Data about therapeutic compliance and clinical outcome (**Table 3**) show that the most of adolescents (70%) followed the therapeutic indications adequately and nearly 60% of adolescents presented a clinical improvement one year later. Results about outcome obtained using the GAF (filled in by the operators) are confirmed by the ones obtained using the YSR (filled in by the patients) (**Table 5**). Looking at the scores before and after the treatment it can be noticed that there is an improvement for each syndrome scale, with a statistically significant p-value <

0.05. There are no significant associations between diagnosis and outcome, suggesting that in developmental age a clinical improvement is more dependent on the therapy and the adherence to it, rather than on the specific psychopathology. Particularly, the results about associations between compliance and outcome (patients who undergo to therapy profiting of it) and between therapy timing and outcome (the longer is the therapy, the more probable is the improvement) suggest that the intervention is efficient when the sessions are attended continuously by the adolescent and for a longer time (**Table 4**). A brief intervention (19% of adolescents came to the centre for less than 3 months) is strictly connected with the phenomenon of drop out. This phenomenon turns out to be more frequent at the beginning of the treatment, and particularly in the first 3 months which is the period dedicated to knowledge and definition of the therapeutic project. The problem of early interruption is significant for the relation with patients' clinical outcome. Between the adolescents who dropped out and those who took part in the whole therapeutic project, there is a statistically relevant difference in terms of clinical evolution, respectively negative and positive. Generally, it must be pointed out the statisticcally relevant relation between therapeutic compliance and clinical outcome: among patients who are compliant there is a significantly larger rate of adolescents who have showed clinical improvement, compared with the non compliant adolescents. This data, moreover, confirms that patients attending the Neuropsychiatric Unit are affected by a psychological and behavioural disease which needs a moderately long period of time to be worked out. On the basis of this consideration it is possible to interpret the result about the diagnosis (**Table 2**): such result, actually, shows that most adolescents are affected by serious psychiatric disorders (psychosis 18%, depression 21% and personality disorders 21%). These psychopathological conditions are confirmed by the results of YSR: looking at scores (borderline and clinic ones) before intervention it can be noticed that the most frequent problems were: withdrawal and social problems (69% and 87%), anxiety and mood disorders (92%), attention problems (97%) (**Table 5**). Attention problem is probably a symptom of anxious and depressive syndromes rather than a symptom of an ADHD (Attention Deficit and Hyperactivity Disorder). This is supported by the fact that attention problems were reduced after intervention (see YSR scores after intervention, **Table 5**) even if the treatment was not specifically designed for ADHD and the timing of therapy was not long enough for that kind of disorder.

Our data suggests that an active participation of the adolescent too contributes to the achievement of a positive result. **Table 4**, actually, shows that an active participation of the patient during the treatment implies a higher probability to obtain a clinical improvement. According to many authors this result confirms that with adolescents, the therapeutic process must be supported by patient's motivation and his/her involvement into therapy dynamics [26-29].

The association between type of intervention and outcome shows that the subjects who got clinically betterare those patients who underwent multiprofessional therapy (**Table 4**) and, particularly, the associations between psychological and educational intervention, (63%), psychological and psychiatric intervention (71%) and the three types together (79%). It must be pointed out that these three types of multiprofessional intervention have a common element that is the focus on the *human relationship*: that is the relation between the patient and the clinician/therapist or with the educational professional. Moreover, these types of interventions require an interprofessional team to integrate different therapeutic actions applied to the same patients. Both a multiprofessional intervention (giving a specific answer to the individual and a answer thanks to the intervention of differ-

Table 4. Results of logistic regression analysis for patients with therapy effectiveness (cases) compared to patients without therapy effectiveness (controls).

	Maximum likelihood estimate	Standard Error	p-value	Odds ratio	95%CI
Therapeutic compliance (ref. 'adequate')	1.751	0.656	0.0076	5.762	1.594-20.829
Type of intervention (ref. 'multiprofessional')	1.432	0.635	0.0242	4.187	1.205-14.544
Timing of intervention ('< 3 months' compated to '> 9 months')	-1.483	0.617	0.0163	0.062	0.009-0.439
Patient's participation to the therapy (ref. 'active')	1.468	0.597	0.014	4.342	1.346-14.002

Likelihood ratio test: $p < 0.0001$. Ref, reference category; CI, confidence intervals.

Table 5. Distribution of patients by Achenbach's scores before and after the intervention [observed frequencies (%)] and paired t-test value with corresponding p-value.

		before (100 patients)	after (94 patients)	paired t-test value	p-value
	normal	31 (31)	66 (70.2)		
Withdrawal	borderline	21 (21)	19 (20.2)	5.61	< 0.0001
	clinic	48 (48)	9 (9.6)		
	normal	41 (41)	67 (71.3)		
Somatic complaints	borderline	40 (40)	25 (26.6)	5.03	< 0.0001
	clinic	19 (19)	2 (2.1)		
	normal	8 (8)	41 (43.6)		
Anxious-depressive problems	borderline	31 (31)	50 (53.2)	9.79	< 0.0001
	clinic	61 (61)	3 (3.2)		
	normal	13 (13)	45 (47.9)		
Social problems	borderline	41 (41)	41 (43.6)	6.43	< 0.0001
	clinic	46 (46)	8 (8.5)		
	normal	40 (40)	64 (68.1)		
Thought problems	borderline	35 (35)	23 (24.5)	3.54	< 0.001
	clinic	25 (25)	7 (7.4)		
	normal	30 (30)	65 (69.1)		
Problems of Attention	borderline	46 (46)	27 (28.7)	6.45	< 0.0001
	clinic	24 (24)	2 (2.1)		
	normal	80 (80)	84 (89.4)		
Delinquent behaviour	borderline	14 (14)	9 (9.6)	2.49	< 0.05
	clinic	6 (6)	1 (1.1)		
	normal	54 (54)	75 (79.8)		
Aggressive behaviour	borderline	27 (27)	17 (18.1)	4.58	< 0.0001
	clinic	19 (19)	2 (2.1)		

ent professionals) and a shared methodology with the possibility of verifying the work done on the individual within a group, contribute to a positive outcome [30,31]. According to that, many studies have just tested the efficiency of multimodal interventions on different psychopathologies such as ADHD, anxiety disorders, suicidal behaviours, conduct disorders, psychosis etc. [32-35].

6. CONCLUSIONS

This study, with the limitation of being a retrospective research, confirms the major efficacy of a multiprofessional integrated approach to the adolescent's psychopathology in comparison with approaches based on a single therapeutic intervention.

A rigid separation among approaches could actually make the operator run the risk of getting a deformed vision of patient's real needs. This approach does not consider the patient as a complex whole and could easily lead to partial and inefficient interventions.

In order to obtain a positive clinical outcome in the treatment of young patients affected by psychiatric disease, it is essential to organize services for adolescents trying to stimulate and support team work, so as to assure a multiprofessional intervention.

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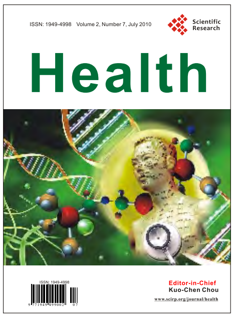

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