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Cox proportional hazard model and its application to data analysis of failure of endodontic equipment

Veronika Bendova^{a,b}, Michal Bumbalek^c, Stanislav Katina^{a,b}

Background and Aims. A very common statistical method used in statistical analysis of medical data is the Cox proportional hazard model (Cox model). The aim of this paper is to describe statistical data analysis as a process beginning with data management, followed by statistical coding and statistical analysis, including survival analysis and the Cox model, and finishing with the interpretation of results. The methods are applied to the data from stomatology – the influence of selected variables on the fracture of endodontic equipment. Statistical analyses were performed in R.

Methods. Exploratory analysis and visualization methods are firstly presented. The survival analysis methods of Kaplan-Meier's product limit estimate, estimate of the cumulative hazard function, and Nair's 95% confidence bands are then calculated. Finally, the conditional hazard is modelled with the Cox model where regression parameters are tested with the Wald test and the proportionality of hazard with the Schoenfeld test.

Results. In the Cox model, covariates influencing the fracture of endodontic equipment, the radius of the dental canal, the size and brand of the endodontic equipment (*Mtwo*, *ProTaper*, *Revo-S*, and *Wizard Navigator*) were used. The regression parameters of the radius of the dental canal and endodontic equipment size proved to be statistically significant. Compared to the reference brand *Mtwo* endodontic equipment, the regression parameters of the *ProTaper* and *Revo-S* drills proved to be statistically significant.

Conclusions. Fracturing endodontic equipment during a root canal treatment often leads to undesirable resting of the tool in the root canal of the patient's tooth, which can lead to less successful endodontic treatment and thus less satisfactory prognosis. The dentist's efforts should allow the use of endodontic equipment for sufficient root canal treatment with minimum risk of fracturing the equipment inside the root system. Removal of the equipment is often very difficult and sometimes fails, resulting in subsequent hermetic closure of the root canal by the root filling not being possible and thereby greatly reducing satisfactory prognosis for the tooth.

Key words: Cox proportional hazards model, R software, Ni-Ti endodontic equipment, *Mtwo*, *ProTaper*, *Revo-S*, *Wizard Navigator*

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INTRODUCTION

The Cox proportional hazards model^{1,2} (Cox model) is one of the most common statistical tools used to analyze medical data. The main advantage of the model is that it has no assumptions for hazard distribution (it is semi-parametric). Its other advantages include simplicity and ease of interpretation of results which have practical use. In the following text, a step by step analysis using the Cox model will be described. In the first part, how to properly determine questions we want to answer using the Cox model and how to convert them into statistical hypotheses for testing will be described. A section on data preparation and exploratory data analysis follows. Methods suitable for visualizing and summarizing data with respect to their type are presented. The Cox model is also introduced, and individual model parameters and assumptions that ensure the correctness of the model are described. Statistical hypotheses of individual regression parameters included in the Cox model are tested, and

correct interpretation of obtained results is focused on. In the second part of the text, the findings are applied to real data where the influence of selected variables on the hazard of Nickel-Titanium (Ni-Ti) endodontic equipment fracture is analyzed. The dataset includes right-censored observations and the time to failure is observed. Data analysis is performed using R software³. Selected steps of statistical analysis are briefly described using R pseudo-code.

MATERIALS AND METHODS

Basic terminology of survival analysis

The notion behind using the Cox model is to model the hazard of occurrence of observed events for a statistical unit in the study, taking into account the time when the observed events occurred on those statistical units. A statistical unit can be, for example, Ni-Ti endodontic equipment. The observed event then may be fracturing

Ni-Ti endodontic equipment. The Ni-Ti equipment is included in the study from the moment of its first use.

From when the statistical unit is entered in the study through to the end of the study, the time to occurrence of the observed event (fracture of the Ni-Ti equipment) is recorded. The following two situations may occur. The observed event either occurs for a statistical unit during the study, or the event does not occur. If no event occurs for the statistical unit during the study, we cannot determine the time of occurrence of the event. Where type of damage other than fracturing occurs and renders the Ni-Ti equipment unusable (such as warping or jamming of the Ni-Ti equipment in the endodontic canal), it is not possible to determine when fracture of the Ni-Ti equipment will occur.

Such data containing incomplete information about the occurrence of an event are called *right censored*. Statistical units which experienced the event are assigned with the status *failure* (denoted as F or 1). Statistical units which did not experience the event are assigned with the status *censor* (denoted as C or 0).

The Cox model is used to model the hazard of occurrence of the observed events in a causal relationship with selected independent variables (covariates). In this example, two types of variable are distinguished. The first type is a continuous variable (e.g., diameter of the Ni-Ti endodontic equipment at the narrowest point). The second type is a categorical variable (e.g., brand of the Ni-Ti endodontic equipment, size of the Ni-Ti equipment determined by the ISO standard). A categorical variable has any finite number of categories, but the number of categories is significantly smaller than the sample size. A special case of categorical variables are binary variables that have only two categories (e.g., two selected Ni-Ti equipment sizes given by the ISO standard, for example, sizes 20 and 25). The binary variable categories are usually denoted as 0 (lower risk group) and 1 (higher risk group).

By using the Cox model, these questions can be answered: Is it possible to explain the increased hazard of occurrence of the event (Ni-Ti endodontic equipment) by the size of the Ni-Ti equipment? Does the use of these brands have any influence in the observed event?

Preparation of materials for analysis

One of the most important steps preceding every data analysis is a systematic review to help gain an overview of the latest developments related to the studied area. This step should lead to correct formulation of questions and statistical hypotheses. It also assures that the Cox model is really the appropriate tool for our data analyses. If we understand the researched domain well, we can also better assess whether the results of the Cox model correspond with previously published findings.

Another part of the preparatory phase is formulation of statistical hypotheses. At least one pair of a null and alternative hypothesis is always defined. The null hypothesis (denoted H_0) is a statement whose validity cannot be rejected or rejected based on the testing approach. An alternative hypothesis (denoted H_1) is a statement that

must be considered when a null hypothesis is rejected. Examples of a null and an alternative hypothesis pair: H_0 : The risk of fracturing Ni-Ti equipment does not vary depending on the brand; H_1 : The risk of fracturing Ni-Ti equipment differs for at least one pair of specified brands. H_0 : The size of the Ni-Ti equipment does not affect its fracture; H_1 : The size of the Ni-Ti equipment affects the fracture of the Ni-Ti equipment.

Preparation of the dataset for analysis and quality control

After collecting all measurements, data should be compiled in a file (or files) of a known type. For analysis in R software, we recommend using a *.csv type file with UTF-8 or Windows-1250 encoding. Before commencing data analysis, quality control must be performed.

First, the column separator (semicolon, vertical bar, tab, etc.) and the decimal separator must be unified in the whole dataset. It is also advisable to check for the presence of missing (NA) values before performing data analysis and unify their coding. Consistency of time, date, or other data formats is also necessary to check.

It is important to note that the presence of such errors in a dataset will lead to bad formatting of the dataset and negatively affect the resulting analysis. Finally, if the data are distributed in multiple datasets, it is advisable to predefine key merging variables that uniquely assign each value to its correct statistical unit after merging the sets.

Exploratory data analysis

After quality control of the data, we can proceed with the data analysis. The dataset is loaded into a statistical software and how many variables and their type present in the dataset are determined. Based on this information, the appropriate methods to visualize and summarize them can be chosen.

To obtain an overview of categorical variables, a table of absolute and relative frequencies should be created. The relative frequencies are presented as estimated probabilities or percentages and visualized using a bar plot of relative frequencies.

For continuous variables, the basic characteristics are computed. The table of characteristics should contain the arithmetic average, standard deviation, median, first and third quartiles, interquartile range, minimum and maximum values, and the skewness and kurtosis. Choosing a histogram and a boxplot⁴ to visualize the data is recommended.

If the dataset is analyzed using the Cox model, it is advisable to also conduct an exploratory data analysis for interactions of the covariates with the variable *status*. It means that the statistical units are divided into *failure* and *censored* groups according to the variable *status*. Consequently, an exploratory data analysis is performed for each group separately. This provides initial insight into how the covariates affect occurrence of the observed event. Based on the results of the analysis, the variables that will be statistically significant in the Cox model and those more likely to not can be anticipated.

Plotting the cumulative hazard function $\Lambda(t)$ and the

survival curve $S(t)$ as part of the exploratory data analysis is also recommended. The survival function is a function that gives the probability that a statistical unit will survive beyond any specified time. It is a non-increasing step function with jumps at the occurrence times of the observed event (fracture) for at least one statistical unit (Ni-Ti equipment). Its values are estimated using the Kaplan-Meier estimate^{5,6}, or the Nelson-Aalen estimate^{6,9}. The Nair's 95% confidence band^{6,10} is also visualized for the survival and cumulative hazard functions. Within the boundaries of Nair's 95% confidence band, the survival function $S(t)$ occurs at any time t with a probability of 0.95. Equivalently, we can visualize the cumulative hazard function $\Lambda(t) = -\ln S(t)$. The cumulative hazard function is defined as the event rate at time t conditional on survival until time t or later. It is a non-decreasing step function with jumps at the times of event occurrence for at least one statistical unit.

With the inclusion of categorical variables in the Cox model, it is advisable to plot the survival functions separately for each category in one graph. By comparing the curves, it can be determined whether the trend of the survival function for all categories is the same (the curves will overlap) or different (the curves will be separated from each other and will not intersect).

Here, it is worth mentioning that a categorical variable with more than two categories is very difficult to interpret in the Cox model. Therefore, prior to commencing construction of the Cox model, it is necessary to convert each categorical variable, let's say with m categories, into m dummy variables. Note that the j -th dummy variable, $j = 1, \dots, m$, is a binary variable whose value is equal to 1 if the statistical unit had a value equal to j , and 0 otherwise. For each statistical unit, the categorical variable can therefore be completely recoded in a way that m categories of the statistical unit create m -set of dummy variables. The binary variable can be interpreted well in the Cox model.

Cox proportional hazards model

After finishing the exploratory data analysis, mathematical modeling can be done. Suppose we have n statistical units in the study. For some statistical units, the observed event occurred ($status = 1$, failure) while for others not ($status = 0$, censored). The variable t describes the time of occurrence of an event, or the right censored time. For each statistical unit, the k values of selected variables (covariates) were also recorded. To represent the k -set of covariates, we use the symbol $\mathbf{Z} = (Z_1, \dots, Z_k)^T$, where Z_1 is the symbol for the first covariate, Z_2 for the second covariate, etc. For covariates $Z_1, \dots, Z_k$, their values are assumed to be time invariant, and that they influence the distribution of time to event occurrence in some way.

The Cox model describes the relationship between the conditional hazard function of the occurrence of the observed event and covariates $Z_1, \dots, Z_k$. The conditional hazard function is denoted $h(t|\mathbf{Z})$, because its shape depends on the k -set of covariates $\mathbf{Z}$ and the baseline hazard function $h_0(t) > 0$, which is the function of the variable t . The Cox model has the form

$$h(t|\mathbf{Z}) = h_0(t)e^{\beta_1 Z_1 + \dots + \beta_k Z_k},$$

where $h(t|\mathbf{Z})$ is the conditional hazard function, $h_0(t)$ is the baseline hazard function, $\mathbf{Z} = (Z_1, \dots, Z_k)^T$ is the k -set of covariates, and $\boldsymbol{\beta} = (\beta_1, \dots, \beta_k)^T$ is the vector of parameters. Parameter β_i belongs to covariates Z_i , $i = 1, \dots, k$.

To get a specific form of the Cox model, it is necessary to estimate the baseline hazard function $h_0(t)$ and the parameters $\beta_1, \dots, \beta_k$. The baseline hazard function $h_0(t)$ is estimated nonparametrically¹¹. The estimation of parameters $\beta_1, \dots, \beta_k$ is based on maximizing the partial likelihood function². In the practical part, parameters $\beta_1, \dots, \beta_k$ are estimated using the **survfit** function implemented in the **survival** package. Here, the values $\hat{\beta}_1, \dots, \hat{\beta}_k$ are assumed estimates of parameters $\beta_1, \dots, \beta_k$. The baseline hazard function $h_0(t)$ and its estimate will not be dealt with here.

We note that $e^{\beta_1 Z_1 + \dots + \beta_k Z_k}$ is not dependent on time t . After substituting concrete values for covariates $Z_1, \dots, Z_k$ and parameters $\beta_1, \dots, \beta_k$, the number by which we multiply the baseline hazard function $h_0(t)$ is obtained.

Suppose now that we have two statistical units, A and B, with a k -set of covariates $\mathbf{Z}_A = (Z_{A,1}, \dots, Z_{A,k})^T$ and $\mathbf{Z}_B = (Z_{B,1}, \dots, Z_{B,k})^T$. The hazard ratio of these two statistical units defined as

$$\begin{aligned} \frac{h(t|\mathbf{Z}_A)}{h(t|\mathbf{Z}_B)} &= \frac{h_0(t)e^{\beta_1 Z_{A,1} + \dots + \beta_k Z_{A,k}}}{h_0(t)e^{\beta_1 Z_{B,1} + \dots + \beta_k Z_{B,k}}} = \\ &= \frac{e^{\beta_1 Z_{A,1} + \dots + \beta_k Z_{A,k}}}{e^{\beta_1 Z_{B,1} + \dots + \beta_k Z_{B,k}}} = e^{\beta_1 (Z_{A,1} - Z_{B,1}) + \dots + \beta_k (Z_{A,k} - Z_{B,k})} \end{aligned}$$

will be a time-independent constant. A special case occurs if the value of the covariates of two statistical units differs in only one variable. Consider two statistical units, A and B, with a k -set of covariates $\mathbf{Z}_A$ and $\mathbf{Z}_B$. Let us assume that the values of all covariates in both statistical units are the same except for the covariate Z_j , i.e., $Z_{A,i} = Z_{B,i}$ for $i \neq j$ and $Z_{A,j} \neq Z_{B,j}$. Now, the hazard of the occurrence of the observed event for statistical unit A and B is compared.

$$\begin{aligned} \frac{h(t|\mathbf{Z}_A)}{h(t|\mathbf{Z}_B)} &= \frac{h_0(t)e^{\beta_1 Z_{A,1} + \dots + \beta_k Z_{A,k}}}{h_0(t)e^{\beta_1 Z_{B,1} + \dots + \beta_k Z_{B,k}}} = \\ &= e^{\beta_1 (Z_{A,1} - Z_{B,1}) + \dots + \beta_k (Z_{A,k} - Z_{B,k})}, \end{aligned}$$

Since $Z_{A,i} = Z_{B,i}$, for $i \neq j$, all differences $(Z_{A,i} - Z_{B,i})$ for $i \neq j$ are equal to zero. The final form of the hazard ratio is

$$\frac{h(t|\mathbf{Z}_A)}{h(t|\mathbf{Z}_B)} = e^{\beta_j (Z_{A,j} - Z_{B,j})},$$

From the previous section, we know that continuous or binary variables are included in the Cox model. So, if the value 1 is denoted for the covariate $Z_{A,j}$ and the value 0 for the covariate $Z_{B,j}$, then the hazard ratio will be

$$\frac{h(t|Z_A)}{h(t|Z_B)} = e^{\beta_j}.$$

The expression e^{β_j} is equal to the constant $e^{\hat{\beta}_j}$ after substitution of estimate $\hat{\beta}_j$ for parameter β_j . Interpretation of the number $e^{\hat{\beta}_j}$ is as follows: The hazard of occurrence of the observed event for the statistical unit with the covariate value $Z_j = 1$ is $e^{\hat{\beta}_j}$ times higher than for the statistical unit with the covariate value $Z_j = 0$.

Specifically, interpretation of the binary variables included in the Cox model may look as follows: The risk of fracturing yellow Ni-Ti equipment (size 20) is 2.6 times higher than that of the red ISO coded equipment (size 25).

If the j -th variable is continuous, we put the arbitrary value z for $Z_{A,j}$ and the value $z - l$ for $Z_{B,j}$, where l is an arbitrary number. The hazard ratio will be

$$\frac{h(t|Z_A)}{h(t|Z_B)} = e^{\beta_j(z-z+l)} = e^{l\beta_j}.$$

The expression $e^{l\beta_j}$ is equal to the constant $e^{l\hat{\beta}_j}$ after substitution of estimate $\hat{\beta}_j$ for parameter β_j . Interpretation of the number $e^{l\hat{\beta}_j}$ is as follows: The hazard of occurrence of the observed event in the statistical unit with the covariate value $Z_j = z$ is $e^{l\hat{\beta}_j}$ times higher than in the statistical unit with the covariate value $Z_j = z - l$.

Specifically, interpretation of the continuous variables included in the Cox model may look as follows: The hazard of fracturing Ni-Ti equipment with a diameter at its narrowest point of 0.95 mm is 0.3 times higher than the risk of fracturing Ni-Ti equipment with a diameter at its narrowest point of 0.94 mm ($z = 0.95$; $l = 0.01$).

Testing the hypothesis on the significance of parameters in the Cox model

The Cox model helps us determine which covariates statistically significantly influence the occurrence of the observed event, expressed by the hazard function, and which do not. From the form of the Cox model

$$h(t|Z) = h_0(t)e^{\beta_1 Z_1 + \dots + \beta_k Z_k},$$

we see that the significance of the i -th covariate Z_i depends on the value of the parameter β_i . With an increasing value of the parameter β_i , the contribution of the entire element $\beta_i Z_i$ to the exponent $\beta_1 Z_1 + \dots + \beta_k Z_k$ increases. Conversely, with a decreasing value of parameter β_i , the contribution of the whole element $\beta_i Z_i$ decreases. If the value of parameter β_i reaches zero, the contribution of the covariate Z_i is also zero.

On the significance level α , for each covariate Z_i , $i = 1, \dots, k$, we test the null hypothesis $H_{0,i} : \beta_i = 0$ (parameter β_i is not statistically significantly different from zero), versus $H_{1,i} : \beta_i \neq 0$ (parameter β_i is statistically significantly different from zero). The hypothesis $H_{0,i}$ can be tested using the Wald test^{6,12}, the score test^{2,6}, or the likelihood ratio test^{2,6}. In the practical part, the null hypothesis $H_{0,i}$

is tested using the Wald test implemented in the **coxph** function from the **survival** package. We determine the null hypothesis decision based on the p -value, which is compared to the preselected significance level α . We usually choose $\alpha = 0.05$.

If the p -value is lower than α , the null hypothesis H_0 is rejected. Parameter β_i is then statistically significantly different from zero and the covariate Z_i significantly influences the occurrence of the observed event.

If the p -value is greater than α , the null hypothesis H_0 cannot be rejected. This means that not enough evidence is available to say that parameter β_i is statistically significantly different from zero. Thus, the covariate Z_i does not have a significant effect on the occurrence of the observed event.

The assumption of hazard proportionality

Before using the Cox model, it is necessary to test the proportionality of hazards assumption. This assumption ensures that the hazard ratio $\frac{h(t|Z_A)}{h(t|Z_B)}$ of two statistical units A and B does not change over time. If this assumption is violated, the Cox stratification model or the Cox model with time-dependent parameters^{6,13} should be used instead of the classic Cox model.

The assumption of proportionality of hazards needs to be tested with respect to each covariate. Graphically, the proportionality of hazards can only be checked for binary variables. For each category $j = 1, 2$, a complementary log-log survival function curve¹⁴ $\ln(-\ln(S(t)))$ is built. Time t is plotted on the x axis. If the proportionality of hazards is met, the curves will be parallel. If the proportionality of hazards is violated, the curves will intersect.

The hazard proportionality test assumes that the parameters of the Cox model $\beta_1, \dots, \beta_k$ and the hazard ratio $\frac{h(t|Z_A)}{h(t|Z_B)}$ remain unchanged over time. The null hypothesis has the form H_0 : Parameters $\beta_1, \dots, \beta_k$ are time-independent (the hazard proportionality is not violated). The alternative hypothesis has the form H_1 : At least one of the parameters $\beta_1, \dots, \beta_k$ is not time-independent (the hazard proportionality is violated).

As part of H_0 , we assume the Cox model with time-independent parameters β_i , $i = 1, \dots, k$,

$$h(t|Z) = h_0(t)e^{\beta_1 Z_1 + \dots + \beta_k Z_k}.$$

As part of H_1 , however, we assume the Cox model with time-dependent parameters $\beta_i(t) = \beta_i + G(t)\theta_i$, where

$$h(t|Z) = h_0(t)e^{(\beta_1 + G(t)\theta_1)Z_1 + \dots + (\beta_k + G(t)\theta_k)Z_k}.$$

Note that both models differ only in $G(t)\theta_i$, where $G(t)$ is a function variable in time t and θ_i is the time-independent parameter.

We rewrite the null and alternative hypotheses in the form $H_{0,i} : \theta_i = 0$ and $H_{1,i} : \theta_i \neq 0$. H_0 is tested using the χ^2 Schoenfeld test^{15,16}. In the practical part, the zero hypothesis about the proportionality of hazards is tested using the **cox.zph** function from the **survival** package. We determine the null hypothesis decision based on p -values.

If the p -value is below the significance level α , the null hypothesis $H_{0,i}$ is rejected. It means that the time-variable member $G(t)\theta_i$ contributes significantly to the parameter β_i , $i = 1, \dots, k$. Parameter β_i is therefore time-dependent $\beta_i = \beta_i(t)$ and the assumption of proportionality of hazards is not met.

If the p -value is greater than the significance level α , the null hypothesis $H_{0,i}$ cannot be rejected. This means not enough evidence is available to say that time variable $G(t)\theta_i$ contributes to the parameter β_i . Therefore, we assume that parameter β_i is time-independent. If all parameters $\beta_1, \dots, \beta_k$ are time-independent, the assumption of hazard proportionality is met.

RESULTS AND DISCUSSION

In the previous section, a theoretical background for data analysis using the Cox model is presented. A preparation step comprising systematic review and data analysis while focusing on visualization and summarization methods for exploratory data analysis is described. We have become acquainted with the Cox model, the interpretations of its results, and a test of the null hypothesis of parameters $\beta_1, \dots, \beta_k$. Finally, methods for testing the assumption of the proportionality of hazards were introduced. All this knowledge will now be demonstrated in a practical example.

Description of the dataset: Fracture of endodontic equipment

A problem for dentists is fracturing endodontic equipment during dental treatment inside an endodontic canal. When this happens, the remainder of endodontic equipment can remain in the tooth, which is very uncomfortable for the patient in terms of subsequent dental prognosis (the endodontic canal space cannot be satisfactorily filled, and so infection can spread). However, if the endodontic equipment is only damaged during drilling (bending, cracking, etc.), the equipment does not remain in patient's tooth. The aim of the study is to compare different types of endodontic equipment in terms of their endodontic treatment life. The data was measured at the Department of Mechanical Engineering at the University of Defense (Brno, Czech Republic) in cooperation with the Institute of Clinical and Experimental Dental Medicine, First Faculty of Medicine, Charles University in Prague and General Teaching Hospital (Prague, Czech Republic).

The dataset contains data on 142 specimens of Ni-Ti endodontic equipment. The equipment was subjected to a stress test simulating load during dental treatment. During the experiment, the equipment was drilled into an endodontic canal simulator made from stainless steel. Drilling ceased when the equipment broke or when other damage occurred to the equipment. A broken endodontic equipment was assigned $status = 1$ (occurrence of event), a damaged endodontic equipment was assigned $status = 0$ (censored). For each piece of endodontic equipment, the time to event occurrence, or the right censored time, was recorded in seconds ($time$). The longest time of break-

ing endodontic equipment was 120 s, the longest time of damage endodontic equipment was 300 s.

For each piece of endodontic equipment, three variables were further monitored. The variable *radius* (Z_1) describes the radius of curvature of the simulated endodontic canal (3 mm or 5 mm). The variable *color* (Z_2) describes the color of the endodontic equipment representing its size in accordance with the ISO standard (yellow – size 20, red – size 25). The variable *type* (Z_3) shows the brand of the endodontic equipment. Four brands of Ni-Ti endodontic equipment¹⁷ were included in the study: *Mtwo* (VDW dental, Munich, Germany), *ProTaper* (Dentsply-Maillefer, Ballaigues, Switzerland), *Revo-S* (Micro-Mega, Besancon, France) and *Wizard Navigator* (Medin, Nové Město na Moravě, Czech Republic).

The variables *color* and *radius* are binary, while the variable *type* is categorical with four categories: *Mtwo*, *ProTaper*, *RevoS* (*Revo-S*), and *WN* (*Wizard Navigator*). We replaced this variable with four dummy variables named *Mtwo*, *ProTaper*, *RevoS*, and *WN* using the **mutate** function implemented in the **dplyr** package. The variable *Mtwo* acquires the value 1 if the endodontic equipment is of the brand *Mtwo*, and the value 0 otherwise, the variable *ProTaper* acquires the value 1 if the endodontic equipment is of the brand *ProTaper*, and the value 0 otherwise, and so on. Thus, the resultant dataset has 9 columns and 142 rows. An overview of the dataset is shown in Table 1.

```
data <- read.delim('endodontic_equipment.csv',
  sep = ';', header = T)
data <- dplyr::mutate(data,
  Mtwo = (type == 'Mtwo')*1,
  ProTaper = (type == 'ProTaper')*1,
  RevoS = (type == 'RevoS')*1,
  WN = (type == 'WN')*1)
```

Table 1. View of the dataset *endodontic_equipment.csv*.

time	status	radius	color	type	Mtwo	Protaper	RevoS	WN
95	1	3mm	yellow	WN	0	0	0	1
51	1	3mm	yellow	WN	0	0	0	1
90	1	3mm	yellow	WN	0	0	0	1
72	1	3mm	yellow	WN	0	0	0	1
48	1	3mm	yellow	WN	0	0	0	1

Based on our knowledge of the subject, the following three aims were set.

Aim 1: Find out if the risk of fracturing endodontic equipment is higher in the endodontic canal with a radius of curvature of 3 mm.

Aim 2: Find out if the risk of fracturing is higher for the size 20 endodontic equipment (yellow color in accordance with the ISO standard).

Aim 3: Find out if the risk of fracturing is the same for all the brands compared.

Exploratory data analysis

We will take a closer look at variables *status*, *radius*, *color*, *Mtwo*, *ProTaper*, *RevoS*, and *WN*. All these variables are binary. For each variable a table of absolute and relative frequencies and a bar plot of relative frequencies is

created. Below is the frequency table (Table 2) and the bar plot for the variable *status* (Fig. 1).

Table 2. Table of absolute and relative frequencies for the variable *status*.

	n	%
Censored	35	24.65
Failure	107	75.35
sum	142	100.00

The *n* indicates the absolute frequency, while the symbol % indicates the relative frequency.

Fig. 1. Bar plot of relative frequencies for the variable *status*. From 142 items of endodontic equipment, fracturing occurred in 75.35% (Failure), while other damage occurred in 34.65% (Censored).

For statistical analysis using the Cox model, performing an exploratory data analysis of interaction of the covariate with the variable *status* is recommended. The results of the interaction analysis provide an initial view of the effect of the covariate on the occurrence of the observed event. For each interaction, it is advisable to compile a table of absolute frequencies and a table of relative frequencies with a summation row and column. The ratio of relative frequencies is visualized in a bar plot. Below is the absolute frequency table (Table 3) and the bar plot of relative frequencies (Fig. 2) for the interaction of variables *radius* and *status*.

Table 3. Table of absolute frequencies for the interaction of variables *radius* and *status*.

	3 mm	5 mm	Sum
Censored	0	35	35
Failure	73	34	107
Sum	73	69	142

Fig. 2. Bar plot of relative frequencies for the interaction of variables *radius* and *status*. Fracture occurred in all endodontic equipment applied to the canal with a radius of curvature of 3 mm. For the canal with a radius of curvature of 5 mm, approximately half the equipment (49.28%) fractured.

In the exploratory data analysis, we also plot the survival function $S(t)$ and the cumulative hazard function $A(t)$ for fracturing endodontic equipment (Fig. 3). To obtain estimates and create figures, the **Surv** and **survfit** functions from the **survival** package were used.

```
data$SurvObj <- with(data, Surv(time, status == 1))
km <- survfit(SurvObj ~ 1, data = data)
plot(km, xlim = c(0, 120), xlab = 't (in s)', ylab = 'S(t)',
     col = 'darkblue', conf.int = F)
plot(km, xlim = c(0, 120), xlab = 't (in s)', ylab = 'S(t)',
     col = 'darkblue', conf.int = F, fun = "cumhaz")
```

We also recommend plotting the survival function curve and cumulative hazard function curve separately for each covariate category of *radius*, *color*, and *type*. By choosing the covariate *type*, the survival function curves for each of the four endodontic equipment brands can be compared. Supplementary Fig. 1 in the Supplementary Material provides graphs of the survival function curves for variables *radius*, *color*, and *type*, including Nair's 95% confidence band.

The survival curve of endodontic equipment applied to a canal with a radius of curvature of 3 mm decreases faster than the survival curve of endodontic equipment applied to a canal with a radius of curvature of 5 mm (see Supplementary Fig. 1, top left). For a while, the survival curve of size 25 endodontic equipment matched course with the size 20 equipment survival curve, but then dropped more sharply (see Supplementary Fig. 1, top right). The fastest decrease in the survival curve is shown by endodontic equipment of the *ProTaper* brand. The second fastest drop in the survival curve is exhibited by endodontic equipment of brands *Revo-S* and *Wizard Navigator*, which match course. However, endodontic equipment of the *Mtwo* brand shows the most gradual course of the survival curve (see Supplementary Fig. 1, bottom).

Fig. 3. Fracture of endodontic equipment: The survival function $S(t)$ (left) and the cumulative hazard function $A(t)$ (right), including Nair's 95% confidence band. The functions $S(t)$ and $A(t)$ were estimated using the Kaplan-Meier estimate.

Analysis using the Cox proportional hazards model

Our aim is to express the relationship between the risk of fracturing endodontic equipment and the *radius*, *color*, and *type* covariates. Since the Cox model will be used for the analysis, the categorical covariate *type* will be replaced by the binary dummy variables *Mtwo*, *ProTaper*, *RevoS*, and *WN*.

The relationship between the dummy variables is such that for the endodontic equipment brand *Mtwo*, the value of the variable *Mtwo* is equal to 1 and the value of the remaining three dummy variables is 0, for the *ProTaper* brand of the endodontic equipment, the value of the *ProTaper* variable is 1 and the value of the other dummy variables is 0, and so on. From this definition, it therefore follows that if the three dummy variables are 0, then the fourth dummy variable must acquire the value of 1. Therefore, it is sufficient to include only three dummy variables in the model for clear determination of the endodontic equipment brand. In our case, the variables *ProTaper*, *RevoS*, and *WN* were chosen. Endodontic equipment of the brand *Mtwo* was selected as a reference brand to which products of the other brands were compared. In general, any endodontic equipment brand can be selected for the reference group.

The Cox model has the form

$$h(t|\mathbf{Z}) = h_0(t)e^{\beta_1 Z_1 + \beta_2 Z_2 + \beta_3 Z_3 + \beta_4 Z_4 + \beta_5 Z_5},$$

where $Z_1 = \text{radius}$, $Z_2 = \text{color}$, $Z_3 = \text{ProTaper}$, $Z_4 = \text{RevoS}$, and $Z_5 = \text{WN}$.

To create the Cox model, the functions **coxph** and **Surv** from the **survival** package were used.

```
model <- coxph(formula = Surv(time, status)
  ~ factor(radius) + factor(color) +
  factor(ProTaper) + factor(RevoS) +
  factor(WN), data = data)
```

Proportionality of hazards test

Before using the Cox model, it is necessary to test the proportionality of hazard assumption. The proportionality of hazards is graphically validated using the log-log survival function curve $\ln(-\ln(S(t)))$. The curve $\ln(-\ln(S(t)))$ is constructed separately for each category of each binary variable. In this case, we plot five graphs with two curves each. The graphs for covariates *radius* and *color* are presented below (Fig. 4), and the graphs of the log-log survival function for variables *ProTaper*, *RevoS*, and *WN* are shown in Supplementary Fig. 2.

```
fit <- with(data, survfit(Surv(time, status) ~ radius))
sfit <- summary(fit)
S3 <- sfit$surv[sfit$strata == 'radius=3mm']
S5 <- sfit$surv[sfit$strata == 'radius=5mm']

plot(sfit$time[sfit$strata == 'radius=3mm'], log(-log(S3)),
  type = 's', ylim = c(-4.5, 1.5), col = 'darkgreen',
  xlab = 't (in s)')
lines(sfit$time[sfit$strata == 'radius=5mm'], log(-log(S5)),
  type = 's', col = 'darkblue')
```

In covariates *radius*, *ProTaper*, and *WN*, the proportionality of hazards was not violated. In covariates *color* and *RevoS*, a slight violation of the hazard proportionality existed. Whether these violations were statistically significant is determined by testing.

At the significance level $\alpha = 0.05$ the null hypothesis $H_0: \theta = 0$ (proportionality of hazards is not violated) is tested against the alternative hypothesis $H_1: \theta \neq 0$ (proportionality of hazards is violated). The proportionality of hazards is also tested separately for each covariate. The function **cox.zph** from the **survival** package was used for testing. The test results are shown in Table 4.

```
cox.zph(model)
```

Fig. 4. Fracture of the Ni-Ti endodontic equipment: The log-log survival function $\ln(-\ln(S(t)))$ for the variable *radius* (left) and the variable *color* (right).

Table 4. Proportionality of hazards test: Summary results table of the function **cox.zph**.

	ρ	χ^2 statistics	p -value
Radius: 3mm	-0.1880	2.8349	0.0922
Color: red	0.0006	0.0000	0.9946
Protaper vs Mtwo	-0.1697	1.8037	0.1793
Revo-S vs Mtwo	-0.1160	1.1641	0.2806
WN vs Mtwo	-0.1210	1.4564	0.2275
Global	NA	3.6275	0.6042

This table includes values of Schoenfeld's coefficients of correlation with the survival function, χ^2 test statistics, and p -values of the hazard proportionality of hazards tests.

Column ρ in Table 4 contains values of correlation coefficients characterizing the degree of dependence between the Schoenfeld residuals and the survival function. Column χ^2 statistics shows the test statistics values for the proportionality of hazards test. In the column p -value, the p -values of the proportionality of hazards test are shown.

Since all p -values are greater than the significance level $\alpha = 0.05$, the null hypothesis $H_0: \theta = 0$ is not rejected for the whole model or any covariate. Thus, the assumption of the proportionality of hazards is fulfilled for the whole model and the individual covariates. The violation of the proportionality of hazards recorded in the graphs of function $\ln(-\ln(S(t)))$ proved to be insignificant.

Cox proportional hazards model

Now, the Cox model created by the function **coxph** will be focused on. The output of the function **coxph** is listed in Table 5.

Column β in Table 5 contains estimates of parameters $\beta_1, \dots, \beta_5$ (denoted as $\hat{\beta}_1, \dots, \hat{\beta}_5$). In column e^{β} , values of exponential function $e^{\beta_1}, \dots, e^{\beta_5}$ are listed. Column $sd[\beta]$ has the standard deviations of estimates $\hat{\beta}_1, \dots, \hat{\beta}_5$.

The z -statistics column contains the test statistics of the significance test for the covariates $Z_1, \dots, Z_5$ in the Cox model. At the significance level $\alpha = 0.05$, we test for each covariate Z_i , $i = 1, \dots, 5$, the null hypothesis $H_{0,i}: \beta_i = 0$ (parameter β_i of the relevant covariate Z_i is not statistically significantly different from zero) versus $H_{1,i}: \beta_i \neq 0$ (parameter β_i is significantly different from zero).

In the p -value column, the p -values of the test of the statistical significance of covariates are shown, while statistically significant p -values are marked in bold. In addition, statistical significance is described by a star notation where the symbol “.” indicates a significance level of less than 0.1, “*” indicates a significance level of less than 0.05, “**” indicates a significance level of less than 0.01, and “***” indicates a significance level of less than 0.001.

Since the p -values for the relevant covariates *radius*, *color*, *ProTaper*, and *RevoS* are lower than the significance level $\alpha = 0.05$, the null hypothesis $H_{0,i}: \beta_i = 0$ for $i = 1, \dots, 4$

Table 5. Cox proportional hazards model: Summary results table of the **coxph** function.

	β	e^{β}	$sd[\beta]$	z -statistics	p -value
Radius: 3 mm	5.1935	180.0952	0.4491	11.5634	< 0.0001***
Color: red	1.2764	3.5836	0.2323	5.4949	< 0.0001***
Protaper vs Mtwo	8.5735	5289.7959	0.7606	11.2728	< 0.0001***
Revo-S vs Mtwo	3.4404	31.1999	0.4411	7.8002	< 0.0001***
WN vs Mtwo	3.1669	23.7342	0.4124	7.6796	0.3357

This table includes estimates of the Cox model parameters, exponential functions of these parameters, and estimates of standard deviations of parameter estimates, test statistics, and the p -values of the significance tests of the parameters.

$i = 1, \dots, 4$, is rejected. Parameters $\beta_1, \beta_2, \beta_3$, and β_4 are statistically significantly different from zero, and therefore the covariates $Z_1 = \text{radius}$, $Z_2 = \text{color}$, $Z_3 = \text{Protaper}$, and $Z_4 = \text{RevoS}$ significantly affect the risk of fracturing the endodontic equipment.

Since the p -value of the relevant covariate WN is higher than the significance level $\alpha = 0.05$, the null hypothesis $H_{0.5}; \beta_5 = 0$ is not rejected. Parameter β_5 is not statistically significantly different from zero and the covariate $Z_5 = WN$ does not significantly affect the risk of fracturing the endodontic equipment compared to *Mtwo* as the reference group.

The conclusions from testing agree with the results of the exploratory data analysis. The survival curves of various categories of covariates *radius* and *color* were noticeably different. Similarly, the survival curves for endodontic equipment of the brands *ProTaper* and *Mtwo* were different and remote from each other. The survival curves for endodontic equipment of the brands *Revo-S* and *Wizard Navigator* had a relatively parallel course, however, the survival curve for the *Revo-S* brand equipment was a little further from the survival curve for the *Mtwo* brand equipment. From the results of the statistical tests, the difference between the *Revo-S* and *Mtwo* survival curves was found to be statistically significant, but the difference between the survival curves of *Wizard Navigator* and *Mtwo* was not.

Interpretation of the Cox proportional hazards model

One of the main advantages of using the Cox model is the practical interpretation of the exponential functions of parameters $\beta_1, \dots, \beta_5$. Therefore, the values from column e^β from Table 5 will be used for interpretation. Based on the analysis carried out using the Cox model, we came to the following conclusions:

1. The risk of fracturing endodontic equipment when drilling the endodontic canal with a radius of curvature of 3 mm is 180.1 times higher than that with a radius of curvature of 5 mm. It follows that the risk of fracturing the equipment during treatment of more curved root canals is significantly higher than a less curved canal, and it is necessary to adapt its time of use and type.
2. Endodontic equipment of size 25 (red) has a 3.6 times greater risk of fracturing than endodontic equipment with size 20 (yellow). Generally, the elasticity is higher for endodontic equipment with lower size applied on a canal with higher radius of curvature, but it also depends on the brand and the producer.
3. The *ProTaper* brand of endodontic equipment has a 5289.8 times higher risk of fracturing than the *Mtwo* endodontic equipment. So, it is appropriate to apply the *Mtwo* brand of endodontic equipment to an endodontic canal with a higher radius of curvature. The elasticity of the *Mtwo* brand equipment is determined by the shape of the cross-section.
4. The *Revo-S* brand of endodontic equipment has a 31.2 times higher risk of fracturing than the *Mtwo* endodontic equipment. The elasticity of the *Revo-S* equipment is also determined by the shape of the cross-section.

5. The *Wizard Navigator* brand of the endodontic equipment has a 23.7 times higher risk of fracturing than the *Mtwo* endodontic equipment. However, the difference between both brands in the risk of fracturing endodontic equipment is not statistically significant.

CONCLUSION

The Cox model is one of the most used and cited models in the field of survival analysis of medical data. In this text, exploratory data analysis, survival analysis, the proportionality of hazards test, the construction of the Cox model, and the correct interpretation of results have been described. The theoretical knowledge listed in the Material and Methods section was subsequently applied to real data. The effect of three covariates on the risk of fracturing Ni-Ti endodontic equipment was observed. The results show that the risk of fracturing endodontic equipment is higher for endodontic equipment with a size of 25 applied to an endodontic canal with a radius of curvature of 3 mm. Endodontic equipment brands can be classified according to the degree of risk of fracturing from lowest to highest as follows: *Mtwo*, *Wizard Navigator* and *Revo-S*, *ProTaper*. It follows that it is necessary to apply endodontic equipment with higher elasticity on an endodontic canal with a higher radius of curvature. The elasticity is determined by the cross-section shape, the conus, edge setting, and alloy type of Ni-Ti endodontic equipment. The dentist's effort should be to use endodontic equipment with a lower risk of damage, that is, with a longer survival time.

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SUPPLEMENTARY MATERIAL

Supplementary Fig. 1. Fracture of the endodontic equipment: Survival function $S(t)$ for the covariates *radius* (top left), *color* (top right) and *type* (bottom).

Supplementary Fig. 2. Fracture of the endodontic equipment: Complementary log-log survival function $\ln(-\ln(S(t)))$ for the covariates *ProTaper* (top left), *RevoS* (top right) and *WN* (bottom).

Sleep apnea prevalence in chronic heart failure patients with severe left ventricle systolic dysfunction

Jiri Hlasensky, Lujza Zikmund-Galkova, Ondrej Ludka

Background. While sleep apnea (SA) might be a modifiable cardiovascular risk factor, recent data suggest that SA is severely underdiagnosed in chronic heart failure (CHF) patients especially with severe left ventricle systolic dysfunction. We therefore investigated the prevalence of SA in these patients.

Methods. We prospectively studied 192 CHF patients with severe left ventricle dysfunction. All subjects were in stable condition and underwent sleep evaluations using Embletta Gold during hospitalization. Using the apnea–hypopnea index (AHI), groups were defined as patients without SA (<5 events/h), mild SA (5–15 events/h), moderate SA (15–30 events/h), and severe SA (≥ 30 events/h).

Results. Using a threshold of $AHI \geq 5$ events/h, SA was present in 75% of patients with CHF and severe systolic dysfunction. Moderate to severe SA was present in 48.4% of them.

Conclusion. There is a high prevalence of SA in patients with CHF with severe systolic dysfunction. Whether diagnosis and treatment of SA will significantly improve outcomes in these patients remains to be determined.

Key words: sleep apnea, heart failure, systolic dysfunction

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INTRODUCTION

Sleep apnea (SA) is highly prevalent in patients with cardiovascular disease and is thought to contribute to the onset and progression of cardiac damage. Pathophysiological connections between chronic heart failure (CHF) and sleep apnea (SA) have already been proven by several studies^{1,2}. Patients with SA have higher cardiovascular morbidity and mortality³ and early diagnosis and treatment of SA should improve not only the quality of life of these patients, but also reduce their morbidity and mortality⁴ with the following reduction of many pathophysiological factors causing development and progression of heart failure⁵. Therefore, we conducted a prospective study of all patients presenting to our hospital with stable CHF to further investigate the prevalence of SA and their types in the patients with CHF with severe left ventricle systolic dysfunction.

METHODS

This study was conducted in accordance with the amended Declaration of Helsinki and was approved by the Ethic Committee of the University Hospital Brno, and all patients provided informed consent.

We prospectively studied 192 patients admitted to the hospital with the diagnosis of CHF with severe left ventricle systolic dysfunction, which were in stable condition for more than 3 months. The most common reason for admission was planned implantation of ICD or CRT-D (61%). The exclusion criteria were: age below 18 and previous continuous positive airway pressure (CPAP)

therapy. All patients underwent complete clinical and biochemical evaluation, ECG and echocardiography at the admission. The disk summation method (modified Simpson's rule) was used for the assessment of left ventricular ejection fraction and was less than 35% in these patients. All subjects underwent limited sleep study using Embletta GOLD device. Outputs were analyzed manually by two investigators blinded to the patient's clinical characteristics. Respiratory events were classified according to the standard criteria of the American Academy of Sleep Medicine. Onset of sleep was recorded at the time at which respiration settled to a rhythmic stable pattern. The end of the recording time was the waking time reported by subjects. Using the apnea–hypopnea index (AHI), groups were defined as patients without SA (<5 events/h), mild SA (5 to 15 events/h), moderate SA (15 to 30 events/h), and severe SA (≥ 30 events/h). We differentiate between obstructive sleep apnea (OSA), central sleep apnea (CSA) and mixed sleep apnea (MSA).

Personal data security was guaranteed by assigning each patient in the study a trial number. All data were recorded in electronic form, and processed using standard descriptive statistics (mean, standard deviation for continuous variables, frequency tables for categorical variables). For the assessment of differences between groups of patients for continuous variables, the Kruskal–Wallis and Mann–Whitney tests were used for non-normal distributions. Categorical variable interrelationships were determined by Chi-square test. Two-tailed *P* values <0.05 were considered as significant, and were corrected for multiple testing.

Table 1. Patient characteristics (categorical parameters).

	All (n=192)	No SA (n=48)	Mild SA (n=51)	Moderate SA (n=43)	Severe SA (n=50)	<i>P</i> ^a
Male gender	83.9	85.4	76.5	79.1	94	0.01
Coronary artery disease	61.9	66.6	60.7	58.13	60	0.002
Atrial fibrillation	41.6	33.3	37.2	48.8	48	0.02
Hypertension	73.4	81.2	68.6	67	74	0.01
Betablockers	85.9	87.5	82.3	88.3	86	0.018
ACE inhibitors/Angiotensin receptors blockers	77	85.4	74	69.7	78	0.001
Diuretics	91.1	87.5	86.2	95.3	96	0.02
Digoxin	21.3	16.6	23.5	25.6	22	0.017
ICD	28.1	43.75	27.4	27.9	14	0.26
CRT-D	33.3	3.3	27.4	39.5	34	0.008

Values are presented as percentage

^a*P*-value of Chi-square test

Table 2. Patient characteristics (continuous parameters).

	All (n=192)	No SA (n=48)	Mild SA (n=51)	Moderate SA (n=43)	Severe SA (n=50)	<i>P</i> ^a
Age (years)	67.7±10.1	67.4±8.2	66.7±9.2	69.8±8.8	66.8±9.8	0.79
Body mass index (kg/m ²)	29.3±5.1	29.7±4.6	30.0±4.9	29.7±5.3	30.3±4.9	0.89
LVEF (%)	29.3±6.2	26.8±4.6	30.7±5.4	26.8±4.5	28.6±6.2	0.56
SBP (mmHg)	125.0±17.9	122.9±14.3	122.9±13.8	126.1±12.6	129.6±16.5	0.53
DBP (mmHg)	76.0±11.0	75.2±8.9	76.5±7.7	75.6±10.6	79.8±9.2	0.59
HR (beats/min)	76.5±14.8	72.5±12.2	77.6±14.4	77.7±13.8	78.35±11.3	0.55

Values are presented as mean, standard deviation

^a *P*-value of Kruskal-Wallis test

Table 3. The distribution of type of SA.

	Mild SA (n=51)	Moderate SA (n=43)	Severe SA (n=50)	<i>P</i> ^a
Obstructive SA	80.4	62.7	40	0.3
Central SA	7.8	9.4	34	0.41
Mixed SA	11.8	27.9	26	0.30

Values are presented as percentage.

^a *P*-value of Chi-square test.

RESULTS

We studied 192 patients. Patients' baseline characteristics are shown in Tables 1 and 2. Data are shown for all subjects, as well as those without SA, and with mild, moderate or severe SA. There were statistically significant differences between the groups in gender, history of hypertension, atrial fibrillation, coronary artery disease, and also medications being taken prior to admission (betablockers, ACE inhibitors, angiotensin receptor blockers, diuretics, digoxin). The vast majority of participants were receiving beta blockers, ACE inhibitors or angiotensin receptor blockers and diuretics.

Using a threshold of AHI≥5 events/h, SA was present in 75% of patients with CHF and severe systolic dysfunction (Fig. 1). The distribution of type of SA in each SA group is shown in Table 3. The prevalence of CSA and MSA increased with increasing severity of SA.

DISCUSSION

We found a high prevalence of previously undiagnosed SA in CHF patients with severe systolic dysfunction. Several studies have suggested that patients with CHF have a high likelihood of SA. However, all consisted of a heterogeneous study population including different severity of systolic dysfunction, diastolic dysfunction, pharmacological and non-pharmacological treatment. Prior studies also used varying thresholds for the diagnosis of SA, and none of them divided patients into groups according to severity of SA (ref.⁶).

Contrary to different studies^{7,8} the most common type of SA in our study was obstructive. But prevalence of CSA and MSA increased with increasing severity of SA.

Since patients with known SA, previously treated by CPAP, were not included in our study, it is probable that we might have slightly underestimated the actual preva-

Fig. 1. Prevalence of sleep apnea in patients with CHF and severe systolic dysfunction of left ventricle.

lence of SA in our study population. On the other hand, the number of patients who are treated for SA in the Czech Republic is still minimal. Differences in diagnostic criteria, types of diagnostic devices, sample sizes, and characteristics of the study population may all affect the determination and prevalence of SA. While SA might be a modifiable cardiovascular risk factor, recent data suggest that SA is severely underdiagnosed in patients with CHF. Deficiencies in diagnosis and hence therapy may be due to logistic and economic considerations. The gold standard for diagnosing SA remains overnight polysomnography, which is expensive and not widely available. Discharging patients from the hospital without a definitive diagnosis of SA would likely delay appropriate intervention. Therefore, sleep evaluation using Embletta Gold device may provide a useful and economical strategy for identifying patients at risk for SA and maybe improving prognosis.

CONCLUSION

There is a high prevalence of SA with most prevalent OSA over CSA and MSA in patients with CHF with severe systolic dysfunction. Whether diagnosis and treatment of SA will significantly improve outcomes in these patients remains to be determined.

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Measurement of blood filling in lower limbs by bioimpedance method

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Background: Cardiovascular diseases are the leading causes of mortality and morbidity in modern society. The autonomic nervous systems innervate blood vessel walls and regulate contraction and wall tension. The head-up tilt test is frequently performed for evaluating sympathetic nervous system function. The filling of the lower extremities during the head-up tilt test is measured by the bioimpedance method. Increased blood volume in the area of interest increases the electrical impedance of this area.

Methods: We used a mathematical fitting model to express the measured data. A B time constant of a decreased bioimpedance signal during a head-up tilt was calculated for the right leg during various time intervals of tilt duration. We optimized the duration in a study protocol based on corresponding measured data and a calculated model. A group of 22 healthy volunteers (11 men and 11 women) participated in the study protocol: the protocol included a 2-minute supine position and 5-minute head-up tilt test.

Results: According to the Mann-Whitney test, there were no statistical differences in the B constant between the groups of men and women. Our results showed the optimal time duration of the head-up tilt test to be about 270 s.

Conclusions: Calculating the time constant for decreased bioimpedance during a head-up tilt test might lead to a simple pre-diagnostic method for performing a non-invasive evaluation of the cardiovascular system.

Key words: bioimpedance, optimization, hemodynamics, tilt test

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INTRODUCTION

Cardiovascular diseases are the leading causes of mortality and morbidity¹. The cardiovascular system loses its efficiency especially with changes in stiffness of large arteries^{2,4}. The sympathetic and the parasympathetic nervous systems innervate vessel's walls and regulate contraction and wall tension⁵. Evaluating of the sympathetic nervous system function is ordinary performed by head-up tilt table testing⁶. Head-up tilting leads to a pooling of blood in the lower limbs, resulting in decreased ventricular filling and arterial blood pressure. The following increase in sympathetic activity and decrease in parasympathetic activity leads to an increase in heart rate^{7,8}. Venous occlusion plethysmography is considered the 'gold standard' for making a non-invasive assessment of blood filling. However, this method is most frequently applied to the forearm^{9,10}.

The aim of this work was to evaluate the vessel's filling in the lower limbs during head-up tilt testing by measuring bioimpedance signals¹¹. The bioimpedance method is non-invasive, non-occlusive and patient-friendly. Measured bioimpedance $Z(t)$ consists of two components: fast changing $\Delta Z(t)$ responds to the impedance of pulsatile blood flow and slow changing $Z_0(t)$ responds to the impedance of tissue and residual blood in vessels¹¹⁻¹⁵. A mathematical modelling of the measured bioimpedance signals was used to find the optimal time interval for head-up tilt testing. Therefore, we used the model¹² for the mathematical description of the measured signals for various time interval of measurement lengths. The dynamic properties of blood distribution can be expressed by a time constant of this

decrease – the B constant¹². The purpose of this optimization is to find such time interval when the results of the measured data are satisfactory and when the signal noise is minimal because of artefacts.

METHODS

Subjects

In total, 22 (11 women and 11 men) healthy, normotensive subjects aged 21-29 years participated in this study. All subjects were non-smokers and were taking no medications. The study was approved by the ethics committee of St. Anne's University Hospital in Brno. All subjects gave their written informed consent prior to participating. They were asked about their age and anamnesis. Weight, height, and arm span were measured by a technician at the beginning of the measurement. Table 1 shows a description of the subjects.

Table 1. A description of the study group (Mean±SD).

	Total	Women	Men
Age [years]	23.8±2.8	24.0±3.4	23.6±2.2
Weight [kg]	69.4±10.5	62.5±7.1	76.3±8.6
Height [m]	1.7±0.1	1.7±0.1	1.8±0.1
Arm span [m]	1.8±0.1	1.7±0.1	1.9±0.1
Systolic BP [mmHg]	109.6±7.9	105.0±5.0	114.2±7.7
Diastolic BP [mmHg]	64.6±7.4	64.5±6.9	64.6±8.1
Pulse pressure [mmHg]	45.0±9.7	40.5±6.2	49.5±10.8
Mean BP [mmHg]	79.6±6.0	78.0±5.6	81.2±6.2

Measurements

Measurements were taken at the International Clinical Research Center at St. Anne's University Hospital Brno, Czech Republic.

We measured a continuous impedance signal $Z(t)$, which was recorded using a Multichannel Bioimpedance Monitor (MBM, ISI CAS, Brno, Czech Republic). This monitor used a four-electrode method to measure the bioimpedance signal¹⁶. We used an alternating sine wave current source $I(t)$ with an amplitude of 1 mA and a frequency of 50 kHz. An electrical source was connected to the standard Ag/AgCl electrodes placed on the right part of the neck (carotid) and the right foot, and two electrodes were placed on the right calf for sensing the voltage $U(t)$ between the electrodes $E+$ and $E-$ (Fig. 1).

Fig. 1. Placement of the voltage sensors and the current electrodes.

A 12-lead electrocardiography measurement (ECG12, ISI CAS, Brno, Czech Republic) and continuous blood pressure by finger photoplethysmographic arterial pressure (Finometer PRO, FMS Systems BV, Netherlands) were measured and recorded simultaneously with the impedance signal. At the beginning of the study protocol, a technician measured blood pressure at the subject's left arm. This measurement was taken with a standard patient monitor.

Experimental protocol and data analysis

We recorded data while the subjects were in a 2-min supine position, followed by a 5-min head-up tilt test at 70 degrees. After that, the subjects were returned to the supine position. Data analyses were performed using Matlab software (R2014b).

We calculated the impedance $Z(t)$ of the desired area of the body (the right calf) between electrodes $E+$ and $E-$ according to Ohm's law:

$$Z(t) = \frac{U(t)}{I(t)} [\Omega], \quad (1)$$

where $U(t)$ is the voltage measured between electrodes $E+$ and $E-$, and $I(t)$ is the current.

The blood volume changes in the lower limbs during the head-up tilt test are reflected by the changes in impedance. The impedance of the blood in the area of interest can be calculated as follows:

$$Z(t) = \frac{\rho(t) \cdot l^2}{V(t)} [\Omega], \quad (2)$$

where $\rho(t)$ is the specific resistivity of the blood forming the hypothetical cylinder in all veins and arteries in the area of interest, l is the length of the segment of the limb and $V(t)$ is the volume¹⁶.

Fig. 2. An example of electrocardiography (ECG), blood pressure (BP) and static impedance $Z_0(t)$. The beginning of the head-up tilt test was marked after the end of the tilt table movement. The figure was created using SignalPlant software (ISI CAS, Brno, Czech Republic) (ref.¹⁷)

We then used a low-pass filter (IIR butterworth filter 0.8 Hz) to obtain low frequency (slow changing) impedance $Z_0(t)$ from the measured bioimpedance signal $Z(t)$, which represents the part of the impedance signal corresponding to blood volume. The tilt test phase was manually selected from the whole study protocol record. The beginning of the head-up tilt test was marked after the end of the tilt table movement. The end of the head-up tilt test was marked in the same way, before returning the subjects to a supine position. An example of the measured signals with a marked impedance signal during the head-up tilt test is shown in Fig. 2.

Based on previous research, we used exponential modelling of $Z_0(t)$ for each subject by the exponential function (3) as follows¹²:

$$Z_{0\text{ model}}(t) = A * e^{-\frac{t}{B}} + C [\Omega], \quad (3)$$

where $Z_{0\text{ model}}(t)$ is a calculated model of the time dependence of electrical impedance in the calf by the exponential function. A is the amplitude of impedance change, B is the time constant of impedance decrease, C is the value of the steady state after the tilt test, and t denotes time.

The model function that was used satisfactorily responded to the time shape of the measured data⁸. We calculated this model for the following time intervals: 0-30 s, 0-60 s, 0-90 s,... 0-300 s. The root mean square error (RMSE) (4) was evaluated between the measured impedance signal and the calculated model for each time interval as follows:

$$RMSE = \sqrt{\frac{\int_0^T (Z_{0\text{ model}}(t) - Z_0(t))^2 dt}{T}} [\Omega], \quad (4)$$

where $RMSE$ is the calculated root mean square error, $Z_{0\text{ model}}(t)$ is a calculated model and $Z_0(t)$ is a measured impedance signal. T is time. Likewise, we compared RMSE and the time constants versus the time interval length.

Statistical analysis

Data are expressed as Mean $\pm$ SD. The Mann-Whitney test for two independent samples was used to compare the time constant B and the RMSE difference between the gender groups while assuming the level of $P < 0.05$ was significant.

RESULTS

The parameters of the model fitting function were computed for each subject and each time interval. The measured impedance signal was visualized with a calculated fitting function. Fig. 3 shows an example of this visualization.

Table 2 summarizes the B constant values and RMSE for all subjects and for each gender group. There was no statistically significant difference between women and men in terms of the calculated parameters ($P > 0.05$ for each time interval).

Fig. 4 shows the standard deviation values of the model B constant calculated for all subjects for each time interval. There are two sharp increases with dramatic decreases that slowly reach a plateau after a time interval of 0-270 s. Fig. 5 shows an increasing trend of RMSE in connection with a longer time interval. A plateau is reached after the 0-270 s time interval.

DISCUSSION

In the present study, we measured the hemodynamic response of healthy young men and women to orthostatic changes during a 70-degree head-up tilt test by the bioimpedance method. Our method provided absolute changes to the measured impedance signal compared with percentage changes in calf volume measured by strain gauge plethysmography¹⁸. A mathematical model¹² was used to calculate the time B constant of the impedance time series (Fig. 3, Table 2). As Table 2 and Fig. 4 show, there is an enormous variance of B constant values during shorter time intervals compared to intervals longer than 0-270 s. Our results indicate a shorter time is needed for the head-up tilt test depending on the RMSE and B constant for all subjects.

Nevertheless, there are a few limitations when using this $Z_0(t)$ time series modelling. The validity of the results depends on the quality of the measured impedance time series. The subject must be strictly in a resting position during the measurement. Any muscle load could distort the impedance signal. The subject's ability to provide a head-up tilt test without changing their standing position

Table 2. Time B constant values and RMSE.

Time interval [s]	B constant [s]			RMSE [Ω]		
	Total	Women	Men	Total	Women	Men
0-30	18.3 $\pm$ 11.7	19.1 $\pm$ 13.8	17.4 $\pm$ 9.8	0.061 $\pm$ 0.083	0.082 $\pm$ 0.111	0.040 $\pm$ 0.035
0-60	529.6 $\pm$ 1669.7	972.9 $\pm$ 2324.7	86.3 $\pm$ 134.2	0.063 $\pm$ 0.064	0.078 $\pm$ 0.083	0.048 $\pm$ 0.035
0-90	2011.8 $\pm$ 1410.9	2327.2 $\pm$ 4488.0	1696.4 $\pm$ 3955.7	0.075 $\pm$ 0.057	0.088 $\pm$ 0.068	0.061 $\pm$ 0.042
0-120	2984.1 $\pm$ 7536.1	1675.6 $\pm$ 4994.0	4292.6 $\pm$ 9516.0	0.082 $\pm$ 0.060	0.100 $\pm$ 0.067	0.064 $\pm$ 0.049
0-150	1199.5 $\pm$ 4368.6	191.6 $\pm$ 184.1	2207.4 $\pm$ 6148.9	0.086 $\pm$ 0.067	0.105 $\pm$ 0.080	0.066 $\pm$ 0.045
0-180	1572.6 $\pm$ 6595.4	175.3 $\pm$ 133.1	2969.8 $\pm$ 9329.3	0.088 $\pm$ 0.068	0.105 $\pm$ 0.085	0.070 $\pm$ 0.042
0-210	311.0 $\pm$ 753.6	165.0 $\pm$ 99.0	456.9 $\pm$ 1065.8	0.088 $\pm$ 0.067	0.105 $\pm$ 0.085	0.072 $\pm$ 0.038
0-240	197.1 $\pm$ 225.3	160.1 $\pm$ 69.4	234.1 $\pm$ 314.2	0.086 $\pm$ 0.064	0.103 $\pm$ 0.081	0.069 $\pm$ 0.038
0-270	169.8 $\pm$ 88.0	163.7 $\pm$ 55.4	176.0 $\pm$ 114.4	0.086 $\pm$ 0.062	0.102 $\pm$ 0.079	0.070 $\pm$ 0.034
0-300	170.3 $\pm$ 58.2	173.2 $\pm$ 48.7	167.4 $\pm$ 68.7	0.089 $\pm$ 0.059	0.104 $\pm$ 0.074	0.075 $\pm$ 0.036

Fig. 3. An example of one fitted subject using a decreasing exponential function. The B time constant is 133.4326 s and RMSE is 0.1749 Ω .

for 5 whole minutes is questionable. Some subjects shift their weight during the tilt test, which leads to very strong signal artefacts. The previous mathematical model is impossible to be use in that case. Also, there are some questions about the impedance signal shape for time intervals longer than 300 s (Fig. 5). We recommend attempting a longer time interval for the head-up tilt test to provide an in-depth investigation into signal shape and compare the current results with future research. However, this might lead to another problem, as previously mentioned; the ability of the subject to sustain a resting position.

CONCLUSION

Visualizing vessels filling in the lower limbs during a head-up tilt test is possible through bioimpedance measuring. A mathematical fitting model¹² was used to model the measured bioimpedance signal during a head-up tilt test

Fig. 4. A standard deviation plot of the model B constant for all subjects.

Fig. 5. The mean of RMSE between the measured impedance signal and the calculated model for all subjects.

using various tilt durations in the time series. The *B* time constant expresses the time of bioimpedance decrease during head-up tilt testing, which corresponds to the same time constant of blood filling in the calf. RMSE between the measured signal and the computed model were calculated. Our results indicate the optimal time (0-270 s) of the head-up tilt test duration depends on RMSE and the *B* constant (total: 169.8 ± 88.0 s, women: 163.7 ± 55.4 s, men: 176.0 ± 114.4).

The results of this research could assist optimizing the duration of study protocols for taking bioimpedance measurements. It is necessary to conduct similar research on an older study group or specific groups of patients. Evaluating the decrease of bioimpedance (the *B* constant) during a head-up tilt test could lead to a simple pre-diagnostic test of vascular complications according to how vessels fill.

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Identification of cytokine secretory site in samples derived from heterogeneous tissue

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Background. The cellular secretome plays a crucial role in cell signaling and communication. Variety of methods have been developed for analysis of secreted substances at a single cell level, but no complex flow cytometric panel was developed for analysis of cytokine secretion in particular cell type in solid tissues.

Methods. Here, we introduced a simple flow cytometry-based tool to identify cytokine secretory site in the most abundant lineages in heterogeneous tissues.

Results. This multiparametric method can be easily adapted for use with other organisms and expanded for identification of different molecules of interest.

Conclusion. Such insight on the cellular secretome at a single cell resolution will enable better understanding of paracrine and autocrine action of secreted substances.

Key words: Interleukin-6, cell secretome, cellular lineages, multiparametric single cell analysis

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INTRODUCTION

The cellular secretome is composed of proteins secreted into extracellular space and transmembrane proteins. Bioinformatic analyses revealed that 39% of the human predicted genes have at least one isoform that is secreted or membrane-spanning¹. It is then obvious that secreted proteins play important role in the cell signaling and communication in multiple ways of action, such as autocrine, paracrine and endocrine. Innovative approaches, enabling analysis of tissues at a single cell level, such as flow cytometry, mass cytometry and single cell RNA-sequencing are shedding completely new light on our understanding of cell-cell interactome and on the role of single cell within the whole environment².

Flow cytometry analysis of secreted molecules was invented decades ago and is widely used in blood samples³. However, this approach is still not commonly used for samples derived from solid tissues, most likely due to complexity of cell lineages present. To overcome this pitfall, we combined surface markers for the most abundant lineages within solid murine tissues and cancers with identification of Interleukin-6 (IL-6) secretory site.

MATERIALS AND METHODS

Cell lines

The murine colon cancer cell line CT26 was acquired from ATCC and cultured in RPMI (Thermo Fisher Scientific/TFS, Prague, Czech Republic), supplemented with 10% FBS (TFS) and 1% penicillin/streptomycin (TFS). The murine prostate cancer cell line RM-1 was a kind gift from Prof. Timothy C. Thompson and was cultured in DMEM (phenol red-free, no glucose, no glutamine, cat. A14430; TSF), 10% FBS, glucose (to final

conc. 1 g/L), 1% GlutaMAX (TFS), 1% sodium pyruvate (Sigma-Aldrich), HEPES (pH 7.5; to final conc. 10 mM; Sigma-Aldrich), and 1% penicillin/streptomycin. Cells were routinely tested for mycoplasma contamination.

Mice and animal experiments

Suspension of 1×10^6 CT26 cells in 100 μ L PBS was injected subcutaneously over the right flank of 8 weeks old BALB/c male mice. Tumors were collected two weeks after inoculation. Suspension of 1×10^6 RM1 cells in 100 μ L PBS was injected subcutaneously over the right flank of 8 weeks old C57Bl6 male mice and tumors were collected three weeks after inoculation. 8 weeks old C57Bl6 males and 10 weeks old, mid-pregnant (E13.5) C57Bl6 females were used for collection of prostate and mammary gland. Mice were obtained from the Laboratory Animal Breeding and Experimental Facility of the Faculty of Medicine, Masaryk University, Brno, Czech Republic. Animal experiments were performed at accredited animal facility, approved by the Academy of Sciences of the Czech Republic (AVCR 2013/13); supervised by the local ethical committee and performed by certified individuals.

Tissue processing

Murine prostate was dissected under a stereomicroscope, minced into 1-2 mm pieces and digested with Papain Dissociation System, as recommended (Worthington, Lakewood, New Jersey, United States). Mammary glands and subcutaneous tumors were dissected and necrotic tumor parts were removed prior to digestion. Glands and tumors were minced into 1-2 mm pieces and digested in Collagenase Type I (Worthington) solution (1 mg/mL) in HBSS (TFS) for 1 hour, followed by short 15 μ g/mL DNase I (Roche, Basel, Switzerland) digestion. Mechanical dissociation was performed before and after enzymatic digestion with GentleMACS

Table 1. Validated antibodies used for flow cytometry.

Antigen	Conjugate	Isotype and clonality	Dilution	Cat.No. (Producer)
CD31	FITC	rat IgG2a, κ clone 390	1:200	178359 (Biolegend)
	isotype control	rat IgG2a, κ clone RTK2758	1:200	400505 (Biolegend)
CD45	Pacific Blue	rat IgG2a, κ clone EM-05	1:12.5	PB-553-C100 (Exbio)
	isotype control	rat IgG2a, κ clone RTK2758	1:12.5	400527 (Biolegend)
CD49f	PerCP-eFluor710	rat IgG2a, κ clone GoH3	1:333	46-0495 (eBioscience)
	isotype control	rat IgG2a, κ clone eBR2a	1:333	46-4321 (eBioscience)
CD140a	Biotin/ PE-Cy7	rat IgG2a, κ clone APA5	1:50	135910 (Biolegend)
	secondary detection	streptavidin PE-Cy7	1:1000	405206 (Biolegend)
EpCAM	APC-Cy7	rat IgG2a, κ clone G8.8	1:200	118218 (Biolegend)
	isotype control	rat IgG2a, κ clone RTK2758	1:200	400524 (Biolegend)
IL-6	APC	rat IgG1, κ clone MP5-20F3	1:80	504508 (Biolegend)
	isotype control	rat IgG1, κ clone RTK2071	1:80	400412 (Biolegend)

Dissociator (Miltenyi, Bergish Gladbach, Germany; programs *m_imp_tumor_02-03*, combined). Cell suspensions were washed several times with PBS, filtered through 70 μ m strainer, counted and processed to extracellular staining.

Flow cytometry and data analysis

1x10⁶ cells were stained in 100 μ L of primary antibody cocktail, followed by secondary detection with PE/Cy7 conjugated Streptavidin (1:1000, cat. 405206, Biolegend) and LIVE/DEAD Yellow stain (1:500, cat. L34959, TFS). All steps were performed for 20 min at 4 °C. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, Prague, Czech Republic), both steps were performed for 15 min at ambient temperature. Next, intracellular antigen was stained for 30 min at 4 °C. Cells were then washed, resuspended in PBS and analyzed. PBS was used as a wash buffer. No stimulator of cytokine production, nor inhibitor of extracellular vesicle release was used. Information related to vendors, conjugated fluorochrome, clonality and dilution is summarized in Table 1. Acquisition was performed on FACSVerser (Becton Dickinson Biosciences/BD, Franklin Lakes, New Jersey, United States) and re-analyzed using FlowJo Software (v10.0.7; TreeStar, Ashland, Oregon, United States). Compensation values for latter multicolor analyses were calculated automatically in FACSuite Software (BD) or FlowJo (TreeStar) from single-conjugate stained UltraComp eBeads (eBioscience, San Diego, California, United States). Gating strategy was as follows: cell aggregates and debris were excluded from analysis based on a dual-parameter dot plot in which the pulse ratio (signal height/y-axis versus signal area/x-axis) was displayed. Dead cells were excluded from analysis by staining with LIVE/DEAD Yellow Fixable Dead Cell Stain, as recommended (TFS). Gates for negativity/positivity were set based on isotype control signal. Preprocessed data were uploaded to and analyzed with Cytobank (Santa Clara, CA, United States), using default settings, except that the number of iterations for viSNE analysis was changed to 1300.

Table 2. Overview of markers and probes used for lineage tracking and identification of IL-6 secretory site.

Probe/Antigen	Denoted use
LIVE/DEAD Yellow	Fixable viability dye
CD31	Marker of endothelial cells
CD45	Marker of leukocytes
CD49f	Marker of basal epithelium and subset of stromal cells
CD140a	Marker of mesenchymal and stromal cells, fibroblasts
EpCAM	Pan-epithelial marker
IL-6	Secreted cytokine

RESULTS AND DISCUSSION

Selection of lineage-specific markers for multiparametric analysis of cytokine secretory site

Solid tissues, as well as tumors, are extremely heterogeneous ecosystems, comprising tens of various cell types. To cover the most abundant lineages within the solid samples, we identified several lineage-specific surface antigens (Table 2). We chose CD31 (PECAM-1) as a surrogate marker for endothelial cells and CD45 as a marker of leukocytes. As most of the tissues contain epithelial cells, we chose EpCAM, a pan-epithelial marker, which can better resolve epithelial lineages in combination with CD49f – basal and luminal epithelial cells, both present in secretory epithelium^{4,5} (Fig. 1). To cover also stromal cells and fibroblasts, we decided to use CD140a (PDGFR α), which can be also combined with CD49f to reveal a distinct subpopulation of stromal cells. Markers and their profile of expression are summarized in Table 2. It is necessary to note that adult stem cells and cancer cells can beside epithelial markers co-express antigens specific for mesenchymal cells and fibroblasts, and only their proper combination can distinguish between tumor and stromal cells⁶.

Fig. 1. Resolution of epithelial lineages within murine prostate and mammary gland using detection of CD49f and EpCAM. Epithelial compartment within secretory organs, such as prostate or mammary gland, contains layers of luminal and basal epithelial cells. These two components can be resolved using CD49f and EpCAM staining. Tissues were dissociated and stained for CD31, CD45, CD49f, EpCAM and viability. Cells positive for CD31 and CD45, as well as dead cells and cell aggregates were excluded from analysis, EpCAM-negative cells represent stroma.

Multiparametric analysis of murine prostate and colon tumor grafts

As an example of complex sample analysis, we decided to identify Interleukin-6 (IL-6) secretory site in heterogeneous tumor tissue. The IL-6 signaling represents important oncogenic pathway in prostate cancer and can be modulated by targeted therapies⁷. We, thus, analyzed its production in tumors derived from extraordinarily aggressive murine prostate cancer cell line RM-1 (ref.⁸), suspected to secrete IL-6. For comparison and to verify the specificity of this anti-mouse IL-6 antibody, we analyzed also murine colon tumors derived from CT26 cell line. While RM-1 cells formed soft, heavily infiltrated subcutaneous tumors, the CT26 cells⁹ formed dense, sarcomatoid tumors after subcutaneous implantation, with low leukocyte infiltration and contained virtually no intratumoral endothelium (Fig. 2A).

For better visualization of high-dimensional cytometry data from this multicolor panel, we used tSNE plots, generated with viSNE algorithm¹⁰. viSNE enables plotting of individual cells in a visual similar to a scatter plot, while using all pairwise distances in high dimension to determine each cell's location in the plot. In the viSNE plots, each cell is visualized as a point in high-dimensional space. Each dimension is a single parameter, in our case the expression level of denoted protein. The viSNE optimization algorithm searches for a projection of the points in high-dimensional space into two or three dimensions, so that the pairwise distance between the points are conserved in high- and low-dimensional space. The low-dimensional viSNE map is then shown as a scatter plot, where a cell's location represents information from

all of the original dimensions, and the cell's color (in our case in the 'pseudocolor' scale) represents the level of expression of denoted antigen.

We revealed three distinct compartments – leukocytes; epithelial tumor cells, derived from RM-1 sample; and stromal tumor cells, derived from CT26 sample (Fig. 2B). Leukocyte infiltration was, again, obvious only in RM-1 tumor. RM-1 cells have epithelial phenotype with mesenchymal features (not shown), which is reflected by co-expression of EpCAM, CD49f and CD140a. RM-1 cells, as well as RM-1-tumor infiltrating leukocytes, produce IL-6 (Fig. 2C). The CT26 tumor cells are fibroblast-like and do not express EpCAM, but they co-express CD49f and CD140a, and formed distinct cell clusters in tSNE plots. As expected, we identified a subpopulation of CT26 cells that do not produce detectable amounts of IL-6 and, interestingly, a subpopulation that most likely produces low IL-6 levels. By simple modifications, this multiparametric panel can further provide a deeper insight into the autocrine and paracrine action of selected cytokine, especially when combined with identification of downstream targets, such as phospho-STAT3 (ref.¹¹).

Thanks to the high availability of selected lineage-specific antibodies for multiple species, this panel can be easily adapted for use with other organisms and expanded for identification of different molecules of interest even by scientists lacking extensive experience with flow cytometry. For further optimizations with tissue of interest we highly recommend the use of validated antibodies. One may further consider the use of extracellular transport inhibitors, such as monensin or brefeldin A, and/or immunotoxins and inducers of cytokine production, such

Fig. 2. IL-6 secretory sites in syngeneic murine prostate and colon grafts. Leukocyte infiltration and intratumoral vasculature of RM-1 and CT26 tumors (A). Distinct cellular compartments, identified with viSNE algorithm (B). Landscape of surface CD45, CD49f, CD140a and EpCAM within these compartments and IL-6 production in dissociated RM-1 (C) and CT26 tumors (D). The endothelial compartment was not assessed due to a limited number of CD31⁺ events. Samples were stained with the whole panel of antibodies. Gates were set based on signal from isotype controls. Dead cells and cell aggregates were removed from analysis.

as phorbol 12-myristate 13-acetate (well-known as PMA), ionomycin or bacterial lipopolysaccharide (LPS), especially in hematogenous lineages¹²⁻¹⁴.

CONCLUSION

We introduced a multiparametric platform, enabling analysis of secreted substances in leukocyte, endothelial, epithelial and stromal compartment of heterogeneous tissue samples.

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Standardized hiPSCs culture – vitronectin coating for cGMP compliant protocol

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Background. Human induced pluripotent stem cells (hiPSCs) hold great promise for disease modeling, drug screening, toxicology testing, regenerative medicine and more. An important task for stem cell research translation into the clinic is culturing cells in completely defined conditions. Currently, many researchers culture hiPSCs on a surface coated with Matrigel®, poorly defined protein mixture of animal origin. Alternatively, vitronectin can be used.

Methods. We cultured two hiPSCs lines in mTeSR™1 cell culture medium on a vitronectin-coated surface and compared their pluripotent markers expression and differentiation capacity in vitro to cells cultured on Matrigel® coated surface.

Results. hiPSCs cultured on both surfaces expressed pluripotent markers and differentiated into all three germ layers.

Conclusion. In this study, we tested culture method in completely defined conditions, thus compatible with current good manufacturing practice (cGMP). We demonstrate that vitronectin is a suitable Matrigel® replacement.

Key words: hiPSCs, cGMP, vitronectin, Matrigel®

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INTRODUCTION

Potential of hiPSCs to become any cell type in the human body and their accessibility give many possibilities of their use. After years of research, there is still a way to go to get hiPSCs routinely to the clinic. One of the problems of all cell-based therapies is the need to standardize every part of the process and to minimize the use of an animal-origin material. hiPSCs are not an exception in this matter.

Traditionally, hiPSCs and other pluripotent stem cells have been cultured on a layer of mitotically inactivated feeder cells¹. Most often mouse embryonic fibroblast (MEFs) have been used, but they can be substituted with other cells types like human fibroblasts². Feeder cells support the growth of pluripotent stem cells by producing growth factors and extracellular matrix³. However, there is a risk of contamination coming from feeder cells⁴. Also, quality of a culture is dependent on a quality of feeder cells, which can substantially vary among batches³. Problems with the use of feeder cells led to the development of multiple feeder-free systems⁵. Combination of complex growth medium and coating tissue culture plasticware with special substrates has proven to be effective in keeping pluripotent stem cells culture in an undifferentiated state⁶. Most commonly, the combination of mTeSR™1 and Matrigel® is used. Matrigel® is a mixture of extracellular matrix proteins produced by mouse sarcoma line⁶. Its components are not clearly defined, and it is of animal origin, which limits its potential.

Braam and colleagues tested various proteins contained in ECM (ref.⁷). They reported that only one of them, vitronectin, substantially supports the growth of hESCs in a defined culture medium. Other substrates were suitable for culture in combination with a conditioned medium but vitronectin was the only defined substrate

supporting stem cells in defined medium (mTeSR™1). Unlike Matrigel®, vitronectin can be produced by recombinant technology in a controlled manner, which makes it suitable for cGMP compatible hiPSCs culture. Growing hiPSCs using mTeSR™1 as medium and vitronectin as the substrate would make clinically applicable hiPSCs yet another step closer to the reality.

In this study, we tested whether recombinant vitronectin in combination with mTeSR™1 supports the growth of hiPSCs and whether hiPSCs cell lines maintain their basic properties while cultured on vitronectin. Using such defined conditions is crucial for translation of hiPSCs culture to cGMP conditions and future clinical use of hiPSCs or their derivatives.

MATERIAL AND METHODS

Cell culture

Two hiPSCs lines derived in our laboratory by Epi5™ Episomal iPSC Reprogramming Kit (Invitrogen) were used (CBIA-19 - reprogrammed from human umbilical vein endothelial cells, passages 21-34 and CBIA-44 - reprogrammed from human peripheral blood mononuclear cells, passages 4-17).

Cell cultures were maintained in 37 °C and 5% CO₂ in mTeSR™1 medium supplemented with ZellShield® (Minerva Biolabs, Berlin, Germany) on either Matrigel® (Corning) or recombinant vitronectin (Gibco) coated tissue culture dishes. The medium was changed daily. The cells were passaged at approximately 80% confluence with EDTA (Invitrogen). 1 h prior to passaging and 1 day after passaging medium was supplemented with ROCK inhibitor Y-27632 (Selleckchem) to reduce apoptosis. During the experiment, all samples were frozen once in 10% DMSO in mTeSR™1 supplemented with 25% KOSR.

Embryoid bodies (EB) formation

After 12 passages in different conditions (CBIA-19 at passage 32 and CBIA-44 at passage 15) cells harvested with EDTA were seeded on low attachment, round bottom 96 well plasticware in density 10 000 cells per well in BPEL medium⁸. The medium was changed after 4 days. One week after seeding formed EBs were either directly transferred to Matrigel[®] or vitronectin coated coverslips or, in a different approach, disrupted by treating with TrypLE (Gibco) for 5 min and then transferred. EBs were then cultured for another week with media exchange 4 days after transfer.

Flow-cytometry

Flow cytometry was used for analysis of surface pluripotency markers with phycoerythrin-conjugated antibodies against SSEA-4 (R&D Systems), Tra-1-60 (Miltenyi Biotec) and Tra-1-81 (Miltenyi Biotec). Samples were measured on a BD FACS Canto II flow cytometer (Becton–Dickinson, Heidelberg, Germany). BD FACSDiva (Becton–Dickinson) and Flowing Software (Cell Imaging Core, Turku Centre for Biotechnology, Finland) were used to analyze the data.

Immunocytochemistry

hiPSCs cultures and EBs were analyzed by immunocytochemistry. Briefly, samples were fixed for 15 - 20 min in

Fig. 1. Pluripotency markers are not compromised by culture on vitronectin.

(A) Overlay histograms from flow cytometry analysis of surface markers (SSEA4, Tra-1-60, Tra-1-81) for both lines and both substrates. Passage numbers 34 (CBIA-19) and 17 (CBIA-44) were used. (B) Summarizing graph of A: percentage of marker-positive cells from flow cytometry, mean of both lines and standard deviation are shown. n=2 (C) Immunocytochemistry of Oct-3/4, Sox, and Nanog in CBIA-19 line (both substrates). Passage numbers 32 (CBIA-19) and 15 (CBIA-44) were used. Scale bar = 40 μ m.

Fig. 2. hiPSCs cultured on vitronectin differentiate into all three germ layers. EBs markers for all three germ layers – ectoderm (TUJ1, Otx2), mesoderm (SMA, Brachyury) and endoderm (FOXA2, SOX17) on CBIA-44 cultured on vitronectin are shown. Scale bar = 20 μ m.

4% paraformaldehyde, permeabilized for 30 min in 0.2% Triton-X (in PBST - 0.1% Tween-20 in PBS), blocked for 1 h with 2.5% BSA (in PBST, all from Sigma-Aldrich) and then incubated with primary antibodies in 4 °C overnight. Then they were incubated with secondary antibodies for 1 h at room temperature. Mowiol®4-88 with DAPI (both from Sigma-Aldrich) was used as an embedding medium and for nuclear staining. Samples were analyzed on Zeiss Axiovert 100S inverted fluorescent microscope (Carl Zeiss, Oberkochen, Germany) on either widefield (hiPSCs cultures) or confocal mode (EBs). Acquarium software was used for acquisition and analysis of data (open source, <http://cbia.fi.muni.cz/projects/acquarium.html>)

For hiPSC testing, antibodies against Oct3/4 (Santa Cruz Biotechnology), Sox2 (R&D Systems) and Nanog (Cell Signaling Technology) were used. Differentiation potential was tested by antibodies against SMA (α -actin, Santa Cruz Biotechnology), brachyury (Santa Cruz Biotechnology), TUJ1 (β 3-tubulin, Santa Cruz Biotechnology), Otx2 (Santa Cruz Biotechnology), FOXA2 (R&D Systems) and Sox17 (R&D Systems). Secondary antibodies conjugated with Alexa-488 (Invitrogen), Alexa-555 (Cell Signaling Technology) or NL-557 (R&D Systems) were used.

RESULTS AND DISCUSSION

We successfully cultured two hiPSCs lines on vitronectin for more than 10 passages. After that, we tested them for pluripotency markers and ability to differentiate into all three germ layers. hiPSCs culture in defined conditions is crucial for many of their applications, including possible clinical use. We did not observe any difference between tested hiPSCs lines, therefore we only show data

of one line for immunocytochemistry analyses (Fig. 1, Fig. 2).

hiPSCs maintain pluripotency markers while cultured on vitronectin

After culturing in different conditions, we tested hiPSCs for several pluripotency markers. Surface markers SSEA-4, TRA-1-60 and TRA-1-81 were analyzed by flow cytometry and expression of transcription factors Oct-3/4, Sox2 and Nanog was verified by immunocytochemistry. Both flow cytometry and immunocytochemistry showed no difference between samples grown on Matrigel® and vitronectin (Fig. 1). These results show that hiPSCs can be cultured in completely defined conditions for prolonged periods of time without differentiating and therefore large amounts of cells can be produced.

Culture on vitronectin does not limit differential potential

Next, we tested whether hiPSCs cultured on vitronectin form EBs composed of cell types of all three germ layers. We have chosen 2 markers for each germ layer. Presence of endoderm was tested by antibodies against FOXA2 and SOX17, mesoderm by SMA and brachyury and ectoderm by TUJ1 and Otx2. Formation of all three germ layers was not compromised with culture on vitronectin (Fig. 2).

Braam and colleagues proved that human embryonic stem cells can grow on recombinant vitronectin in mTeSR™1 (ref.⁷). Rowland and colleagues showed successful hiPSCs culture on vitronectin, but the vitronectin used was isolated from human plasma⁹. In both studies, mechanical passaging by colony dissection was used. Such passaging technique is time-consuming and dependent on skilled personnel. Using gentle dissociation agents like EDTA (ref.¹⁰) for cell detachment is more appropriate.

In research of both embryonic and induced pluripotent stem cells, Matrigel® is up to date still prevalent substrate used for feeder-free culture^{11–17}. Matrigel® is not suitable for clinical applications because it is not defined, and it is produced by an animal cell line which poses a risk of pathogens and non-human molecules transmission. For cGMP compatible protocol, controlled production of a precisely defined substrate must be used. Recombinant vitronectin meets these criteria.

CONCLUSION

Vitronectin in combination with mTeSR™1 medium supports the growth of hiPSCs in an undifferentiated state without compromising their differentiation capacity and therefore it can replace the most commonly used substrate, Matrigel®. Matrigel® is produced by mouse cell line and its components are not chemically defined. Defined conditions for hiPSCs culture can be used for a standardized method of hiPSCs culture with lower variability, which is crucial for many applications including production of cGMP grade hiPSCs, thus enabling the clinical use of hiPSCs or any hiPSCs-derived cell types.

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Comparison of radiation exposure, contrast agent consumption and cost effectiveness between computer tomography and 3D rotational angiography of the left atrium to guide catheter ablation in patients with atrial fibrillation

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Background. Catheter ablation of complex atrial arrhythmias guided by 3D models of a left atrium (LA) derived from CT/MRI or 3D rotational angiography of the heart (3DRA) is currently a common practice. Our aim is to compare radiation dose, consumption of a contrast agent and cost of these methods.

Methods. From 10/2012 to 10/2015, either 3DRA (157 patients using the X-ray system Philips Allura FD 10) or computer tomography (CT) (157 patients using 64-slice CT GE Lightspeed VCT) of the LA was performed in patients undergoing complex atrial arrhythmias ablation. All procedures were monitored for average effective radiation dose, dose of the contrast agent and analysis of a financial expenditures.

Results. The average effective radiation dose for 3DRA was 2.11 ± 0.392 mSv compared to 10.52 ± 2.093 mSv for CT. The contrast agent consumption was 22.2 mg and 48.2 mg of iodine for 3DRA and CT, respectively. Total price per procedure is 123€ for 3DRA and 161€ for CT.

Conclusion. Consumption of the contrast agent and radiation burden for LA 3DRA is significantly lower in comparison to most currently used CT scan methods. Cost of both methods is comparable, however, 3DRA is slightly less expensive than CT.

Key words: radiation risk, 3D cardiac rotational angiography of the left atrium, computer tomography, complex atrial arrhythmias, catheter ablation of arrhythmias

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INTRODUCTION

Atrial fibrillation (AF) is the most common supraventricular arrhythmia and, in recent years, catheter ablation of AF has become the most frequently performed electrophysiology (EP) procedure¹. Currently, complex ablations are performed under the guidance of 3D electroanatomical mapping (EAM) systems creating 3D non-fluoroscopic maps of the left atrium (LA) (ref.²). In everyday clinical practice, there are two basic systems used for invasive EP (CARTO, Biosense Webster, and EnSite Velocity, St. Jude Medical).

A standard method used for pre-ablation LA imaging is based on contrast-enhanced computer tomography (CT) (ref.³). Nevertheless, 3D X-ray model-guided LA mapping provides objective information about real anatomy being an valuable clinical tool⁴.

3D Rotational Angiography (3DRA) represents a new alternative to cardiac CT. 3DRA is safe, feasible⁵ and fully comparable with CT image quality⁶⁻⁸. It was developed as a tool for left atrium imaging, however, it can be used for visualization of several other cardiac and adjacent extracardiac structures such as left and right ventricle^{9,10}. Noteworthy, esophagus imaging with CT is safe and feasible¹¹, but clinical usability is questionable due long-term mobility of the esophagus¹². On the contrary, 3DRA could be useful in the imaging modality during left atrial

arrhythmias radiofrequency ablation (RFA) because of short-term mobility of the esophagus¹³. Moreover, the advantage of this technique is associated with a favourable logistics, time-effectiveness (peri-procedural LA model generation), reduction of radiation dose and amount of the contrast agent. Importantly, final 3DRA- and CT-supported RFA result is identical in terms of non-fluoroscopic 3D electroanatomic maps creation, direct fusion/integration with 3D EAM system^{4,7}, and/or direct fusion/integration of a 3D model with live fluoroscopy¹⁴.

However, both imaging methods have a clinically relevant impact on a patient regarding radiation and contrast agent. Moreover, economic aspect should be also taken under consideration. The objective of this study is to compare radiation dose, consumption of contrast agent and costs of these methods in patients undergoing pre-RFA left atrium 3DRA and CT imaging.

MATERIALS AND METHODS

Patient population

We performed a retrospective study enrolling 314 patients referred for catheter ablation of complex atrial arrhythmias between October 2012 and October 2015. All patients underwent either CT or 3DRA imaging of the left atrium. Patients with a history of iodine allergy or with

Fig. 1. Comparison of the 3DRA of the left atrium (A,C) and CT of the left atrium (B,D) from one patient. A,B – lateral view, C,D – anteroposterior view

impaired renal function (MDRD / Modification of Diet in Renal Disease/ of less than 45ml/min/1.73m^2) were excluded from the study.

Rotational Angiography Imaging

3RDA imaging was carried out using the X-ray system Allura Xper FD 10 (Philips Medical Systems Inc., Best, The Netherlands). A basic principle of the 3DRA is based on contrast agent injection (Ultravist 370, Bayer Pharma AG, Berlin, Germany) to the atrium and acquisition of rotational image. After opacification of the LA and pulmonary veins, the C-arm was isocentrically rotated over 240° (120° right anterior oblique to 120° left anterior oblique) over 4.1 s with an X-ray acquisition speed of 30 frames per second. The patients were in a lying position during rotational imaging with natural position of the arms along the body and normal breathing.

Pigtail catheter was introduced to the LA over the transseptal sheath Agilis NxT 8,5F. Left atrium isocentering was achieved from antero-posterior and left-lateral X-ray projections.

In aim to achieve optimal imaging parameters, right ventricular temporary pacing (230 beat per min) was introduced with subsequent reduction of cardiac output and drop in blood pressure followed by disappearance of a pulse waveform at the distal finger phalanx of the right upper extremity (Saturation sensor, Philips IntelliVue MP-20, Philips, Eindhoven, The Netherlands.) Afterwards, contrast agent was injected (amount 60 mL, velocity of injection 15 mL/s) and, with a delay of 2 seconds, we commenced the rotation of C-arm⁷. Application of contrast agent was carried out with standard power injector (Mark V, Medrad Inc., Indianola, PA, USA) (ref.⁸). Figure 1 presents an example LA 3DRA and CT imaging.

Fig. 2. Comparison of the average effective radiation dose and average dose of contrast agent between 3DRA and CT of the left atrium.

At the end of the rotational angiography procedure, data was automatically transferred from the Allura X-ray System to the EP Navigator Workstation (EP Navigator 3.0, Philips Healthcare, Best, The Netherlands). The 3DRA model of LA and pulmonary veins anatomy was automatically reconstructed using the standard algorithms of the EP Navigator Workstation.

CT imaging

A CT scan was performed few days before ablation using non-ECG-gated protocol on a 64-slice CT (GE Lightspeed VCT, General Electric, Fairfield, USA). CT parameters included: 120kV, 800mAs, collimation of 63×0.625 mm, and spiral pitch factor of 0.98. Image reconstruction was performed on a 512×512 pixel array. Contrast agent was administered through a peripheral vein (Ultravist 370, Bayer Pharma AG, Berlin, Germany). During imaging, patients had to have their arms up whilst holding the breath. Afterwards, data was copied into CD and reconstructed during LA RFA procedure using EP Navigator Workstation (EP Navigator 3.0, Philips Healthcare, Best, Netherlands).

Radiation exposure, dose of contrast agent, cost effectiveness

For all 3DRA procedures we determined the average radiation dose presented as a dose area product (DAP, mGycm^2) and consumption of the contrast agent. For CT procedures average radiation dose was determined as a dose length product (DLP, mGycm). The average

Table 1. Baseline characteristics of study group.

	3DRA of the left atrium n=157	CT of the left atrium n=157	<i>P</i>
Age, mean±SD	59.42±9.72	59.87±10.45	1.000
Males, n (%)	117 (74.77%)	113 (71.52%)	0.626
LVEF, mean±SD (%)	57.26±7.04	57.11±8.18	1.000
Left atrium diameter, mean±SD (mm)	44.23±5.25	44.82±5.73	1.000
BMI, mean±SD (kg/m ²)	29.82±9.14	29.49±8.29	1.000
Structural heart disease, n (%)	29 (18.47%)	31 (19.75%)	0.679
Hypertension, n (%)	94 (59.87%)	99 (63.06%)	0.224
Atrial fibrillation, n (%)	143 (91.08%)	139 (88.53%)	0.254
Paroxysmal atrial fibrillation, n (%)	98 (68.53%)	98 (70.50%)	0.770
Persistent atrial fibrillation, n (%)	45 (31.47%)	41 (29.50%)	0.784
Atypical left atrial flutter, n (%)	12 (7.64%)	13 (8.28%)	0.240
Focal left atrial tachycardia, n (%)	2 (1.27%)	3 (1.91%)	1.000

BMI – body mass index, LVEF – Left ventricle ejection fraction

Table 2. Cost summary of LA 3DRA and CT imaging.

Financial costs/€	X-ray system Allura Xper FD 10 + injector + workstation EP Navigator	CT GE Lightspeed VCT + injector + workstation HP WORKSTATION X28200
Purchase price	881 327	1 252 538
Maintenance and service/year	0	93 364
Total depreciation/15 min	22	42
Consumables	89	108
Salaries	12	11
Total cost	123	161

radiation dose was converted to an effective dose (ED) using appropriate converting factor. For 3D rotational angiography where the dose was measured in DAP units a converting factor of 0.18 mSv Gy⁻¹cm⁻¹ was used¹⁵. For CT, where the dose was given in units of DLP, a converting factor of 0.017mSv mGy⁻¹cm⁻¹ was applied¹⁶.

In the same group of patients we compared the quantity of the contrast agent. Taking under consideration that CT scans were performed at different sites using various types and concentrations of contrast agent, the doses of the contrast agent were standardized and converted to mg of iodine.

Analysis of the financial expenses was conducted by the Economic Department at the University Hospital of St. Anne, Brno illustrating conditions that applied to most hospitals in Czech Republic in 2015. Technical fees and wages of individual workers (physicians, biomedical engineers, technicians and nurses) were included into the analysis. Technical fee covered consumables (contrast agent and other small supplies) and depreciation of individual devices. Depreciation of equipment constituted the main part of the cost of the performance was calculated from the cost of equipment and prices for service. For angiography, the Allura FD 10 service is included in the price of the device. Oppositely, for CT scan equipment the price of a service is 93364€/year. Lifetime of both devices is calculated at 5 years and depreciation is calculated for a 8-h working week. Turnaround time for both technologies was set at 15 min. The prices are converted to Euros using the exchange rate as of November 2014 (27.58 CZK/€).

Image integration, ablation procedures

The LA 3DRA model was automatically integrated with the live fluoroscopy¹⁴. We used 3D models of the LA as a guidance for 3D electroanatomical map generation in form of (1) synchronised projection of 3DRA model and 3D EAM system or (2) direct fusion of 3D models with 3D electroanatomical map^{4,7}.

Ablation procedures were performed in a standard manner using irrigated tip catheter guided by 3D EAM system, EnSite Velocity (St. Jude Medical, St. Paul, MN, USA).

Statistical analysis

Statistical analysis of clinical data set was performed using STATISTICA software StatSoft, Inc. (2013), version 12, www.statsoft.com. The statistical analysis was performed by the Kolmogorov-Smirnov test of normality and by the unpaired two sample Student's t-test. The significance level value for both types of statistical analysis was set to value 0.05.

RESULTS

In the period from March 2013 to October 2015 a total of 157 patients were examined with multislice cardiac CT with segmentation of LA 3D model.

In the period from October 2012 to October 2015 a total of 157 LA 3D rotational angiographies were performed

in patients with complex atrial arrhythmias, in most cases atrial fibrillation.

Characteristics of patients are summarised in Table 1. Study population consisted of 230 (73.2%) males, and the mean age was 60 (± 9) years. In both groups there was a similar number of patients with structural heart disease. Moreover, individuals were characterized by slightly enlarged left atrium and normal left ventricle ejection fraction. There was no significant statistical differences between 3DRA and CT group.

Radiation exposure, dose of contrast agent, cost effectiveness

The average radiation dose for LA 3DRA was $DAP=11746\pm 2178$ mGycm².

The average radiation dose for LA CT was $DLP=619\pm 123$ mGycm.

Average consumption of contrast agent for LA 3DRA and CT of was 60 mL and 124.5 mL, respectively.

When comparing the average ED of radiation burden we showed a statistically significant reduction of radiation for 3DRA compared to the CT scan (2.11 ± 0.392 mSv vs. 10.52 ± 2.093 mSv, respectively, $P<0.001$). Data are shown in Fig. 2.

The average dose of the contrast agent used in LA 3DRA and CT imaging was 22.2 mg and 48.4 mg of iodine, respectively. Dose of the contrast agent in 3DRA was significantly lower (Fig. 2)

Total price per procedure is 123€ for 3DRA and 161€ for CT. For details see Table 2.

Catheter ablation guided by 3DRA and CT of the left atrium

All patients with the 3DRA model of LA were ablated with the support of direct overlay 3D model and 2D fluoroscopy. 7 patients with CT and 9 patients were ablated under the guidance of 3D model directly fused with 3D EMA map. Rest of patients were ablated with the support of 3D EnSite Velocity system with LA 3D model superimposed on the 3D EAM map.

DISCUSSION

The modern 3D X-ray imaging methods make the catheter ablations of complex atrial arrhythmias easier and safer. Nevertheless, both methods are expensive and have considerable medical consequences due to radiation and contrast agent application.

In our study, we demonstrated a significantly lower radiation burden and consumption of contrast agent in 3DRA compared to CT imaging method. In published studies, the ED for 3DRA was an average of 2.3 ± 0.3 mSv compared to 19.5 ± 3.1 mSv for CT models^{8,17,18}. An explanation for the significantly higher dose for CT models of the left atrium from the cited works vs. CT scanning in our patients is based on the imaging protocol. In the cited studies, retrospective ECG-gated CT was used, whereas in our cohort we applied ECG-non-gated protocol leading

to less radiation exposure. Thorning et al.¹⁹ measured an average ED of 13.45 mSv for retrospective ECG-gated protocol evaluating pulmonary vein anomalies with CT, while for ECG-non-gated protocol only 6.1 mSv. In the study by Ector et al.²⁰, the difference in average ED was significant higher when using the retrospective ECG-gated vs. non-gated preprocedural cardiac CT (3.17 ± 5.2 mSv vs. 4.4 ± 3 mSv, respectively, $P<0.001$)

Significantly lower consumption of the contrast agent for 3DRA in our study correlates with published results. In the smaller studies comparing 3DRA and CT models of the left atrium, the average quantity of contrast agent used in 3DRA was almost half the amount of contrast agent used in CT (73 mL vs. 120 mL) (ref.^{8,7,17,18,21}).

A lower radiation dose and lower consumption of the contrast agent in 3DRA compared to the CT is relative. Noteworthy, due to intensive development of the most advanced CT imaging, modern CT devices have significantly lower radiation burden and consumption of the contrast agent. In recent years, several studies have been published showing decreased radiation dose and consumption of contrast agent, below the average characteristics of 3DRA. In 2010 Blanke et al.²² published results of LA CT imaging using prospective ECG-triggered sequential dual-source CT scan (Somatom Definition, Siemens) carried out before catheter ablation. The mean ED was 1.1 ± 0.3 mSv and 3.0 ± 0.5 mSv (body mass index [BMI]-dependent tube voltage set on 100 or 120kV).

Yang et al.²³ described the first use of low dose 320-row CT Aquilion ONE, Toshiba for LA and pulmonary veins imaging. The ED was 1.90 ± 0.19 mSv and 3.83 ± 0.31 mSv, respectively (BMI-dependant tube voltage of 100kV or 120kV). In addition to a significantly reduced ED less than half the amount of a contrast agent was used compared to older CT devices (40 and 50 mL according to BMI).

In Czech Republic the economic cost for 3DRA is lower than for CT (Table 2). The difference of the total cost is 39 €, which is 24% of the price of the CT scan. Kristaselis et al.²¹ in 2011 published a comparison of costs for both methods in Federal Republic of Germany. The authors calculated the price of one scan for 3DRA 91-95 € and 126-151 € for CT. In this case, the difference in cost is slightly greater and it constitutes 28-37% of the price of the CT imaging. This is mainly due to the mismatch in costs. In the study of Kritselis et al., angiography costs were very low, while CT expenditures doubled in comparison with our devices. The total costs are difficult to compare; the overall technical fee in our department is 111 € for 3DRA and 151 € for CT, thus, slightly higher than those presented by Kriatselis et al. This is due to the fact that the depreciations in the mentioned study are calculated for 6 years and based on a 10-hour working week, which make the costs lower. If we calculated the cost of both centres according to the same formula, the result would be comparable. The question to consider is what would be the cost of these analysis in Germany if they counted the price of labour of the staff. In our calculations the price of labour is negligible (24 € for

3DRA and 47 € for CT). It is a specific characteristic of Central European post-communist countries, where the cost of labour in healthcare services is underestimated. It is quite possible that after accounting for the cost of labour in Germany, the total cost would be completely different. Comparison of costs of individual methods between Czech and Germany is very difficult and can be misleading. However, we can conclude that in our settings and settings of Western Europe, the technical fee for 3DRA is comparatively lower than for CT. Moreover, in Czech Republic settings, the same applies to the total cost, including the cost of labour.

CONCLUSION

Consumption of the contrast agent and radiation burden for 3DRA of the LA is significantly lower than most currently used CT scan methods.

The advantage of a lower X-ray dose and contrast agent when comparing 3DRA and cardiac CT is relative and with new generation of CT devices both methods might be comparable.

Cost of both imaging methods is comparable, however, 3DRA is slightly less expensive than CT.

Comparing financial expenditures between countries is very difficult due to differences in calculation methodology.

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Feasibility and safety of right and left ventricular three-dimensional rotational angiography for guiding catheter ablation of ventricular arrhythmias

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Background. Three-dimensional rotational angiography (3DRA) of the heart is an imaging technique that displays the left atrium and adjacent structures during catheter ablation of atrial fibrillation. The aim is to evaluate the feasibility and safety of 3DRA for imaging the right and left ventricles of patients undergoing catheter ablation of ventricular arrhythmias.

Methods. From 8/2010 to 6/2015, 35 patients underwent 3DRA of the right (20 patients) or left ventricle (15 patients) with a Philips Allura FD 10 X-ray system using a direct protocol. The success rate of the 3D model, as well as the procedure times and complications of 3DRA, was evaluated, and the 3DRA model was compared with ventricular computer tomography (CT).

Results. The overall 3D model success rate was 91.4%. The 3D models were graded as excellent for 65.7% of patients and as useful for 25.7% of patients. The imaging success rate was slightly higher for the right ventricle than for the left ventricle (95%, 86.7%, respectively). The times required to perform 3DRA of the right and left ventricle were 12.5 $\pm$ 2.1 min and 14.7 $\pm$ 2.8 min, respectively. There were no significant differences between 3DRA and ventricular CT.

Conclusion. Ventricular 3DRA allows the easy and safe creation of 3D models of the cardiac ventricles. The success rate is comparable to the success rate of the 3DRA for imaging the left atrium. There was no difference in imaging quality between the two ventricles. 3DRA models of the ventricles are comparable with CT models of the ventricles.

Key words: 3D rotational angiography, left ventricle, right ventricle, catheter ablation of arrhythmias, ventricular arrhythmias, image integration

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INTRODUCTION

Catheter ablation guided by 3D electroanatomical mapping¹ (CARTO (Biosense Webster, Diamond Bar, CA) and EnSite Velocity (St Jude Medical, St Paul, MN)) is now the method of choice for treating complex atrial and ventricular arrhythmias^{2,3}.

The creation of electroanatomical maps based on 3D models of the left atrium or cardiac ventricles developed via computed tomography (CT) to guide ablation procedures is common^{4,5}.

Three-dimensional rotational angiography (3DRA) of the heart was developed to image the left atrium during catheter ablation of atrial fibrillation⁶⁻⁸ and represents a new alternative to cardiac CT imaging^{9,10}. Models of other cavities and structures, such as the esophagus^{11,12} or cardiac ventricles^{13,14}, can be acquired and used during ablation.

Our objective was to evaluate the feasibility and safety of 3DRA for the creation of models of the left and right ventricle in patients undergoing catheter ablation of ventricular arrhythmias.

MATERIAL AND METHODS

Patient population

This retrospective study enrolled 35 consecutive patients who were referred to our center for catheter ablation of ventricular arrhythmias from August 2010 to June 2015. All patients underwent right or left ventricular 3DRA, and 18 patients also underwent ventricular CT. A study regarding a portion of this group of patients was published previously¹⁴.

Rotational angiography imaging

Imaging was carried out with a Philips Allura Xper FD 10 X-ray system (Philips Medical Systems Inc., Best, the Netherlands) using a protocol described previously¹⁴. The contrast agent (a total of 85 mL of contrast agent was administered at a velocity of 20 mL/sec) was administered into the right ventricle (RV) or left ventricle (LV), and rotational images were acquired (the C-arm was isocentrically rotated 240° over 4.1 s using an X-ray acquisition speed of 30 frames per second).

Ventricle isocentering was achieved using two X-ray projections with a maximally raised flat panel.

Before rotational angiography, cardiac output was reduced via rapid ventricular stimulation at a frequency of 200–220/min.

Fig. 1. Data acquisition and right ventricle (RV) 3D model segmentation. (A) Cardiac output via rapid stimulation was documented based on a decrease in saturation, (B) Segmentation of the 3D model using raw data. Shown are the XYZ sections of the 3D rotational angiography data, with the RV colored in green. (C, D) Rotational angiography of the right ventricle, RAO 104 (C), RAO 10 (D). (E, F) The final rendered model of the right ventricle, RAO 100 (E), RAO 40 (F).
LAO - Left oblique view, RAO - Right oblique view

After rotational angiography, data were automatically transmitted from the X-ray system to an EP Navigator workstation (EP Navigator 3.1, Philips Healthcare, Best, the Netherlands), where 3D models of both ventricles were manually segmented, (Fig. 1). These 3DRA models were automatically integrated with live fluoroscopic images (Fig. 2 and 3).

CT imaging

CT imaging was performed using an ECG non-gated protocol on a 64-slice CT scanner (GE Lightspeed VCT, General Electric, Fairfield, USA). The CT parameters were as follows: 120 kV, 800 mA, a collimation of 63x0.625 mm, and a spiral pitch factor of 0.98. Image reconstruction was performed on a 512x512 pixel array. Contrast was administered through a peripheral vessel.

Qualitative and quantitative image analysis

Qualitative analysis. The rotational angiography imaging results were independently assessed by two expert physicians. The rotational angiography results for each patient were assessed in 23 projections (right anterior oblique (RAO) 55° to left anterior oblique (LAO) 55°, in steps of 5°). The images were graded on a scale of 1–3 as follows: (1) not diagnostic, or characterized by indistinguishable ventricular contours, non-identifiable bodies or unrecognizable ventricular outflow tracts (VOTs); (2) useful, or characterized by blurred ventricular contours, missing or unclear ventricular apex, and poorly visible VOT-aortic/pulmonary valve junctions; and (3) excellent, or characterized by clear ventricular contours, easily identifiable ventricular bodies and ventricular outflow tracts, and identifiable aortic or pulmonary valve-ventricular out-

Fig. 2. Integration of the 3D models of the right (A and B) and left (C and D) ventricle with fluoroscopic images, LAO 30 (A), RAO 39 (B), RAO 39 (C), LAO 41 (D).

RAO - Right anterior oblique projection, LAO - Left anterior oblique projection

flow tract junctions. In the event of disagreement between the two reviewers with respect to angiogram 'grading', the images were re-assessed by both reviewers, and a consensus was reached. This method was modified from that previously described by Tang et al.¹⁵

Quantitative image analysis. The images of the right and left ventricle that were acquired via 3DRA were compared to those acquired via CT. Using the raw data stored in the EP Navigator workstation, we compared the subvalvular diameters of the outflow tracts and the diameter of the aortic/pulmonary valve bulbus measured via 3DRA with those measured via CT (see Fig 3).

Image integration and ablation procedures

All ablation procedures were performed with the support of the 3D ventricular models generated via 3DRA, which were merged with live fluoroscopic images.

We supported the ablation procedures using synchronized projections of the 3D X-ray model and the 3D electroanatomical map or by directly merging the 3D X-ray model with the 3D electroanatomical map.

The ablation procedures were performed in a standardized manner. Idiopathic ventricular extrasystoles originating from the right ventricular outflow tract (RVOT) were conventionally ablated with standard 4-mm tip ablation catheters with the support of the 3D models generated via 3DRA, which were integrated live fluoroscopic images. Left ventricular outflow tract (LVOT) arrhythmias and ventricular arrhythmias caused by structural heart disease were treated with irrigated tip catheters with the support of the 3D electroanatomical mapping system EnSite Velocity.

Statistical analysis

Statistical analysis was performed using STATISTICA software (data analysis software system), StatSoft, Inc. (2013), version 12, www.statsoft.com. Data were analyzed using descriptive statistics. Differences between the two groups (CT vs. 3DRA) were evaluated by Spearman's rank correlation coefficient, and confidence intervals were reported. The two methods were compared using the Wilcoxon-Mann-Whitney U two-sample rank-sum test. The significance level was set to 0.05.

Fig. 3. Comparison of CT and 3DRA raw data measurements. (A, B) Picture of the body of the right ventricle and pulmonary valve, as well as part of the pulmonary artery, A - 3DRA, B - CT, (C, D) Picture of the body of the left ventricle and aortic bulbus, as well as part of the ascending aorta, C - 3DRA, D - CT.

3DRA - 3D rotational angiography, CT - Computed tomography

Fig. 4. Integration of the 3D model of the left ventricle and the 3D electroanatomical mapping system EnSite Velocity, with tagged ablation points on the surface of the left ventricular outflow tract (yellow and white dots).

A - Left oblique projection, B - Right oblique projection

Table 1. Patient characteristics.

Patient characteristics	3DRA of the right ventricle	3DRA of the left ventricle	Total
Number of patients	20	15	35
Age	52.55 +/- 16.42	65 +/- 16.35	57.89 +/- 16.32
Males	11 (55%)	8 (53.33%)	19 (54.29%)
EF of LV	57.95 +/- 8.22	56.53 +/- 8.22	57.34 +/- 8.11
Body mass index	27.92 +/- 4.48	26.98 +/- 4.6	26.36 +/- 4.48
Structural heart disease	0	2 (13.33%)	2 (5.71%)
Hypertension	7 (35%)	7 (46.67%)	14 (40%)
Idiopathic RVOT arrhythmias	20	N/A	N/A
Idiopathic LVOT arrhythmias	N/A	13	N/A
Structural heart disease arrhythmias	N/A	2	N/A

RVOT - right ventricular outflow tract, LVOT - left ventricular outflow tract

Table 2. Duration of individual 3DRA steps.

Duration of individual 3DRA steps	3DRA of the right ventricle	3DRA of the left ventricle	<i>P</i>
Average total time from the introduction of the pigtail to the end of segmentation in EP Navigator (min)	12.5 +/- 2.1	14.7 +/- 2.8	0.036
Average time for data exportation from EP Navigator to the 3D mapping system (min)	NA	4.8 +/- 0.9	
Average total procedure time (min)	131.5 +/- 58.1 †	221.5 +/- 72.2 † (387.0 +/- 43.0*)	†0.002
3DRA share in total procedure time (%)	9.5	6.6 (3.8*)	

* Ablations of ventricular arrhythmias in patients with structural heart disease, n=2

† Ablations of ventricular arrhythmias in patients without structural heart disease, n=33

Table 3. Success rate of 3DRA.

Success rate of 3DRA	3DRA of the right ventricle n=20	3DRA of the left ventricle n=15	3DRA of both ventricles n=35	<i>P</i>
Excellent 3DRA	14 (70%)	9 (60%)	23 (65.7%)*	
Useful 3DRA	5 (25%)	4 (26.7%)	9 (25.7%)	
At least useful (excellent + useful)	19 (95%)*	13 (86.7%)*	32 (91.4%)	*0.048
Not diagnostic 3DRA	1 (5%)	2 (13.3%)	3 (8.6%)	

3DRA - three-dimensional rotational angiography

Table 4. 3DRA versus CT.

Characteristic	N	Spearman corr. coeff.	Wilcoxon test (Z score)	<i>P</i>
Aortic bulbus diameter	5	0.9	0.674	0.50
Subvalvular LVOT diameter	5	0.7	0.405	0.69
Pulmonary valve diameter	12	0.874	1.647	0.10
Subvalvular RVOT diameter	12	0.881	1.530	0.09

RESULTS

From August 2010 to June 2015, 35 patients underwent right or left ventricular 3DRA before catheter ablation of ventricular arrhythmias. Twenty patients underwent also underwent cardiac CT.

The average radiation dose for all ventricular 3DRA procedures was 10875.4 +/- 3318.4 mGy/cm². The average radiation dose for right ventricular 3DRA was 9638.2 +/- 3318.4 mGy/cm², and the average radiation dose for

left ventricular 3DRA was 12731.25 +/- 2399.9 mGy/cm². The average radiation dose for all catheter ablation procedures was 22200.3 +/- 11488.3 mGy/cm². The average radiation dose for right ventricular arrhythmia ablation was 17680.7 +/- 11488.3 mGy/cm², the average radiation dose for LVOT arrhythmia ablation was 27675.7 +/- 8905.5 mGy/cm², and the average radiation dose for LV structural arrhythmia ablation was 38367.3 +/- 8561.1 mGy/cm²

Patient characteristics

Of the 35 patients enrolled in this study, 20 underwent 3DRA of the RV, and 15 underwent 3DRA of the LV. Five patients who underwent 3DRA of the LV underwent CT of the LV, and 13 patients who underwent 3DRA of the RV underwent CT of the RV. Most patients were ablated for idiopathic ventricular extrasystoles originating from the RVOT and LVOT. Three patients had diminished EFs, two patients had structural heart disease (ischemic cardiomyopathy after MI), and one patient with idiopathic LVOT extrasystoles had a diminished EF due to arrhythmia-induced cardiomyopathy.

Rotational angiography image acquisition and qualitative and quantitative image assessment

The mean systolic and diastolic blood pressures at the start of rotational ventricular angiography were 132.67 $\pm$ 17.01 mmHg and 78.54 $\pm$ 13.04 mmHg, respectively. The times required to perform right and left ventricular 3DRA were 12.5 $\pm$ 2.1 min and 14.7 $\pm$ 2.8 min, respectively. Given the small number of patients in both groups, this difference appeared to be significant ($P=0.036$). Total procedure time was significantly longer for ablations involving the left ventricle ($P=0.002$) than for ablations involving the right ventricle. For details, see Table 2.

Qualitative image assessment. The overall success rate of the protocol (excellent and useful models) was 91.4%. The 3D volume reconstructions were graded as excellent in 65.7% of patients and as useful in 25.7% patients. The vast majority of the models classified as useful were missing the apex of the ventricle, which was cropped due to imperfect isocentering. The examination results were useless in three patients, and a system failure occurred in one patient, as the loss of the connection between the power injector and the pigtail catheter resulted in the ventricle not filling with contrast. The resulting model did not anatomically correspond to the visualized ventricle and therefore could not be used in two patients. The success rate of 3DRA of the RV was slightly higher than the success rate of 3DRA of the LV (95% vs. 86.7%). Given the small number of patients in both groups, this difference appeared to be statistically significant ($P=0.048$). After excluding the technical error (disconnected connecting tubes in one case of failed 3DRA) the difference between the success rates of right and left ventricular 3DRA was negligible (95% vs. 92.8) ($P=0.759$). Additional details can be found in Table 3.

Because manual segmentation was performed, the resulting models had somewhat uneven surfaces, but the sizes and shapes of the images completely matched those of the displayed ventricles. Reassessments of anatomical accuracy were performed throughout each procedure, and no discrepancies were reported.

Quantitative image assessment. The datasets of the 3DRA and CT images of the 5 patients who underwent 3DRA of the LV and the 12 patients who underwent 3DRA of the RV (one patient who underwent CT of the RV underwent unsuccessful 3DRA of the RV) were compared. Using the Wilcoxon–Mann–Whitney U two-sample rank-sum test, we failed to reject the null hypothesis that

there is no difference between the two groups at the 0.05 significance level (Table 4).

Catheter ablation with the support of 3DRA

All radiofrequency ablations were performed in the standard manner and were without complications. For catheter ablation of idiopathic RVOT arrhythmias, fluoroscopy and the 3D model were the only imaging methods used. For all ablations of LVOT extrasystoles and arrhythmias caused by structural heart disease, we used the 3D electroanatomical mapping system EnSite Velocity and the 3D model of the ventricle generated by 3DRA to create 3D electroanatomical maps.

In two patients, integration of the resulting 3D image of the LV and the electroanatomical map generated using EnSite Velocity was accomplished using the aortic bulbus and coronary ostia as fiducial points (Fig 4).

DISCUSSION

Three-dimensional rotational angiography of the cardiac ventricles proved to be a simple and reliable method of imaging the cardiac ventricles. The success rate of creating an applicable model using the direct protocol was 91.4%. Orlov et al. noted that their success rate for 3DRA of the right ventricle was 87.5% (ref.¹³), which is comparable to the success rates of the direct left atrial protocols used to image the left atrium via 3DRA. Tang et al. reported a success rate of 95.7% using this left atrial protocol¹⁵. Kriatselis et al. reported a success rate of almost 100% (ref.¹⁶). The success rate of ventricular 3DRA noted in this study was not significantly different from the success rate noted in our cohort of 238 patients who underwent catheter ablation of atrial fibrillation and were examined via 3DRA of the left atrium using the direct left atrial protocol (91.4% vs. 94.54%, $P=0.894$) (ref.¹²).

Not diagnostic or useful images were attributed to the learning curve associated with ventricular 3DRA, as it is a new imaging method. Splitting the patients into two groups according to the dates of their procedures showed that the probability of a not diagnostic or useful result was 38.9% (18 patients, 7 suboptimal results) during the first half of the study and 23.5% (17 patients, 4 suboptimal results) during the second half of the study. For example, early in the series, poor isocentering or problems with image cut-offs caused by improper isocentering resulted in the generation of suboptimal images

The main concern associated with the use of 3DRA models of cardiac cavities is imaging quality. As CT models are considered the gold standard for imaging cardiac cavities, the most reliable means of verifying the accuracy of a 3DRA model is to compare it with a model created using CT data. Several studies have compared models of the left atrium generated using 3D rotational angiography to models generated using CT imaging^{9,6,15}. Comparisons between these atrial models were based mostly on measurements of the ostia of the pulmonary veins and showed that there was a very strong correlation between the models generated by each modality. Using similar methods

in our small group of patients, we noted a very strong correlation between the left ventricular models generated using 3DRA and CT data¹⁴.

An equally important issue is the clinical benefit of using 3D ventricular models. In our cohort of 516 patients who were examined with 3DRA of the left atrium during radiofrequency ablation of atrial fibrillation, the average time from the introduction of the pigtail catheter to the end of segmentation was 8.47 min¹². Despite the need to use manual segmentation when creating the ventricular model, the total procedure times for each ventricle were only slightly longer (12.5 +/- 2.1 min for right ventricle; 14.7 +/- 2.8 min for left ventricle) than that for the left atrium. The manual segmentation time of the left ventricle was slightly longer than the manual segmentation time of the right ventricle, probably because the former procedure was more complicated due to the presence of the aortic bulb and the coronary artery stems; however, from a practical point of view, the difference between the two manual segmentation times was negligible.

We observed contrasting results when evaluating the impacts of ventricular 3DRA on the total duration of and radiation dose associated with the procedure. For the aforementioned group of patients who were referred for atrial fibrillation ablation¹², 3DRA imaging accounted for only 4.5% of the total procedure time and 33.6% of the total radiation exposure. In the case of 3D models of the left ventricle, the impacts of 3DRA on procedure time and radiation exposure were comparable. In the case of catheter ablation of left ventricular arrhythmias in patients with structural heart disease and patients with idiopathic ventricular extrasystoles originating from the LVOT, 3DRA imaging accounted for 3.8% and 6.6% of the total procedure time and 33.2% and 46% of the radiation exposure, respectively. However, in the case of ventricular arrhythmias originating from the RVOT, the impact of 3DRA was less favorable, as 3DRA imaging accounted for almost 10% of the total procedure time and 54.5% of the total radiation dose. Given the relatively large amount of time required to perform 3DRA and the considerable amount of radiation exposure associated with the procedure, which is comparable to or worse than that associated with 3DRA of the left atrium^{6,9,12}, the use of this method to support ablation of ventricular arrhythmias should be carefully considered, and the benefits of its use should be weighed against the possible risks of its use. This method may be recommended for guiding catheter ablations, for which CT-generated heart cavity models are commonly used. For ablation of RVOT extrasystoles, however, routine use of this method is questionable.

Study Limitations

Several limitations need to be addressed. First of all, we were unable to automatically segment and reconstruct the 3D models of the ventricles, as automatic segmentation is available only for reconstruction of the left atrium. The 3D models of the ventricles must be manually segmented, which is somewhat time consuming and requires an experienced operator. This limitation may discourage other centers from routinely employing 3D rotational an-

giography to visualize additional cardiac and non-cardiac structures. Second, the size of the standard cardiology flat detector (10 x 10 inches) is not appropriate for patients with higher BMIs, and the use of this flat detector results in suboptimal isocentering and image cut-offs, which most commonly affect the apex. We also believe that it is impossible to determine the exact contour of the endocardium during rapid ventricular pacing because the contrast-enhanced appearance of the ventricle is the same during both systole and diastole.

CONCLUSION

3D rotational angiography of the ventricles allows the easy and safe periprocedural creation of 3D models of the cardiac ventricles, which are useful for guiding catheter ablation of ventricular arrhythmias. The 3DRA and CT models of the ventricles are comparable. There were no differences in image quality between the right and left ventricle.

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Detailed analysis of the relationship between the left atrium and esophagus in patients prior to catheter ablation of atrial fibrillation: an analysis using 3D computed tomography

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Background. Damage to the esophagus during radiofrequency ablation of atrial fibrillation can result in fatal atrio-esophageal fistula. Our aim is to describe the relationship of the left atrium (LA) and esophagus with a focus on contact of the posterior wall (PW) of the LA and the esophagus.

Methods. From 9/2011 to 8/2012, CT of the heart was performed in 56 patients undergoing catheter ablation of atrial fibrillation using CT GE Lightspeed VCT 64 by nongated standard protocol. Positions of the esophagus to the LA, width of the esophagus and contact with the PW of the LA were determined.

Results. The most frequent position of the esophagus was behind the left side of the LA. The average width of the esophagus was 16.0 ± 4.1 mm, and the average distance between the PW and esophagus was 4.8 ± 1.6 mm. On average, 50 ± 4.11 mm of the PW was in close contact with the esophagus. The average length of the fat pad between the esophagus and LA was 9.1 ± 5.5 mm in the upper part of the LA and 15.8 ± 7.3 mm at the bottom. Significantly longer contact occurred behind the right part of the LA.

Conclusions. The most common esophageal position is behind the left side of the LA. The esophagus is located in close contact by approximately 2/3 of the length and 1/4 of the width of the PW of the LA. The esophagus is more adjacent to the upper part of PW of the LA.

Key words: CT of the heart, left atrium and esophagus imaging, relationship of the left atrium and esophagus, catheter ablation of atrial fibrillation, atrioesophageal fistula

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INTRODUCTION

Catheter radiofrequency ablation (RFA) of atrial fibrillation is currently the most effective, widely used therapeutic method. In recent years, it even became the most common ablation in the treatment of cardiac arrhythmias¹. As an invasive, interventional procedure it is connected with a number of risks and potential complications². One very serious, but fortunately rare, complication is atrioesophageal fistula resulting from a close anatomical relation of the posterior wall (PW) of the left atrium (LA) and the esophagus³. When this complication occurs within a few days after ablation, communication between the left atrium and esophagus emerges due to thermal damage to the wall of the left atrium and the esophagus with very serious consequences for the patient (a series of ablations can occur in the posterior wall of the left atrium during catheter ablation of atrial fibrillation, and the most common are circular lesions around the ostium of the pulmonary veins). Although atrio-esophageal fistula is rare with an incidence rate of 0.04% (ref.²), it is the cause of almost 16% of deaths in connection with catheter ablation of atrial fibrillation⁴ and is fatal in 70-80% of cases^{4,5}. Atrio-esophageal fistula was first described in connection with the perioperative surgical radiofrequency ablation⁶, however, it soon appeared in the first reports of atrioesophageal fistula during percutaneous radiofre-

quency catheter ablation for atrial fibrillation⁷. Several works describe, in various ways, the relationship of the left atrium and the esophagus on anatomical preparations³ or from CT data⁸⁻¹⁰.

Our aim is to describe, in detail, the relationship between the posterior wall of the left atrium and the esophagus with a focus on objectification of the area of close contact between these structures.

METHODS

Patients

This retrospective study enrolled a group of 56 consecutive patients referred for catheter ablation of atrial fibrillation and examined those patients using a multislice CT of the heart and thorax from September 2011 to August 2012. The study received approval from the ethics committee of our institution.

CT imaging

A CT scan was performed before the procedure using the ECG nongated protocol on a 64-slice unit (GE Lightspeed VCT, General Electric, Fairfield, USA). The CT parameters included: 120 kV, 800 mAs, collimation of 63×0.625 mm, and a spiral pitch factor of 0.984. Image reconstruction was performed on a 512×512 pixel array.

Axial images were reconstructed with a slice thickness of 0,625 mm in 0.5 mm increments. The spatial resolution of the images is 0.574x0.574x0.625 mm. A contrast agent was administered through a peripheral vein; the amount of contrast was 100-150 ml (Ultravist 370, Bayer Pharma AG, Berlin, Germany or Iomeron 400, PNG Gerolymatos AEBE, Kryoneri - Athens, Greece). During the procedure, the patients held their arms up while holding their breath.

The procedure was performed a few days before catheterization. The data were burned onto a CD-ROM, and during the procedure, a 3D reconstruction of the left atrium using a workstation EP Navigator (EP Navigator 3.1, Philips Healthcare, Best, The Netherlands) or software of the 3D electroanatomical navigation system (EnSite Velocity, St. Jude Medical, St. Paul, MN, USA) was created from the acquired data.

Fig. 1. (A) CT image from endovascular anteroposterior projection. Posterior wall of the LA was defined (red lines) by a superior line formed by the most superior points of the roof of the LA. The medial line tangential to the most medial (right-sided) points of the ostia of the right-sided PVs, a lateral line that was tangential to the most medial (left-sided) points of the ostia of the left-sided PVs, and an inferior line formed by the posterior mitral annulus or the most inferior points of the bottom of the LA, (B) Methodology of assessment of esophageal position, posteroanterior view, (C) Measurement in sagittal plane, the different levels in which the widths of the esophagus and the distance esophagus - LA were measured in the transverse plane, yellow lines 1-5, measurement of the depth of the LA (anteroposterior diameter), orange line, measurement of the height of the LA (superoinferior diameter), green line, measurement of the contact of the esophagus and LA, area of close contact is highlighted in red, superior segment of fat pad in yellow color, inferior segment in blue, (D) Measurement in the transversal plane, width of the LA (transverse diameter) blue line, width of the posterior wall of the LA, red line, width of the esophagus, green line, measurement of the distance of the esophagus and LA, yellow line. LSPV - left superior pulmonary vein, LIPV - left inferior pulmonary vein, RSPV - right superior pulmonary vein, RIPV - right inferior pulmonary vein, LAA - LA appendage, MA - mitral annulus.

Image evaluation

The obtained data were measured and evaluated offline at the workstation EP Navigator V 3.1 by one investigator (ZS). The intraobserver variability was $6 \pm 3\%$.

To define the posterior wall of the left atrium, we used a modified methodology from Lemola et al.⁹. The posterior LA was outlined by (1) a superior line formed by the most superior points of the roof of the LA; (2) the medial line, which was tangential to the most medial (right-sided) points of the ostium of the right-sided PVs; (3) the lateral line, which was tangential to the most medial (left-sided) points of the ostia of the left-sided PVs; and (4) an inferior line formed by the posterior mitral annulus or the most inferior points of the bottom of the LA (see Fig. 1B). In contrast to the work of Lemola et al., our description of the posterior wall included the ostia of the pulmonary veins, as in some patients the esophagus may be located in an extreme position behind the ostium of the left or right sided pulmonary veins. The position of the esophagus against the PW of the LA was evaluated for each patient.

We performed a segmentation of the 3D model of the LA and esophagus, and in the coronal plane (anteroposterior projection), we evaluated the left-right position of the esophagus to the PW of the LA. To determine the position of the esophagus to the atrium, we used a modified method described by Kottkamp et al.¹¹. To assess the esophageal position, Kottkamp et al. used a grid structure on the CT model of the left atrium. We modified this method and divided the posterior wall of the left atrium and adjacent pulmonary veins into five vertical columns, A – E from left to right. Columns A and E were laterally behind the ostia of the pulmonary veins, column C was in the middle of the left atrial posterior wall, and columns B and D were in the intermediate position. The course and location of the esophagus behind the left atrium was evaluated and was described by one of the columns. In the case of the oblique course of the esophagus intersecting multiple columns, we determined the position of the esophagus according to the column where the largest part of the esophagus lies. As a second option, we evaluated the simplified version of the description of the occurrence of the esophagus behind the left or right side of the left atrium as previously described by Cury et al.⁸.

In the transverse and sagittal planes, we evaluated the width of the esophagus, the size and shape of the left atrium and the contact of the esophagus with the LA posterior wall. A fat pad between the LA and esophagus was identified by an abrupt change in the signal density.

Measurement included:

In the transverse plane

1. The width of the esophagus in five equidistant levels - at the level of the upper and lower borders of the PV of the LA, in the middle, and in the upper and lower quarters of the PV of the LA. Each level of the measurement was numbered 1-5 from top to bottom.

2. The width of the posterior wall of the left atrium - the distance of ostia of the left and right inferior pulmonary veins. This measurement was approximated to

a certain extent, but the exact width measurement of the posterior wall was difficult to perform because an ostia of pulmonary veins could not be clearly determined. Our proposed measurement is easy and feasible, and the ostia of pulmonary veins were included into the width of the posterior wall. Thus, width of the PW covers the entire area which can be in contact with the esophagus.

3. The width of the left atrium (transverse diameter, TD) - the widest place in the transverse section in the same plane as the measurement of the width of the posterior wall.

4. Depth of the left atrium (anteroposterior diameter, APD) - the largest distance between the anterior and posterior walls of the left atrium in the sagittal section.

5. The height of the left atrium (superoinferior diameter, SID) - the largest distance between the top and bottom of the left atrium perpendicular to the depth of the left atrium in the sagittal plane.

6. Contact of the esophagus with the LA posterior wall measured in the transverse plane at the same levels as in the measurement of the width of the esophagus. We measured the distance from the lumen of the left atrium to the lumen of the esophagus. If the esophagus had collapsed, with no apparent luminal air density, then we determined the lumen as a half of the anteroposterior size of the esophagus.

In the sagittal plane

7. The length of the posterior wall of the left atrium measured from the transition of the LA roof into the posterior wall to the bottom of the left atrium or mitral annulus at the point of contact of the esophagus with the LA.

8. Length of close contact of the esophagus and posterior wall of LA, which we defined as the area where the esophagus is very close to the posterior wall of the left atrium, and between these structures, it was less than 1 mm of fat pad.

9. Length of fat pad - area of contact between the LA and esophagus in the upper and lower wedge-shaped part (upper and lower segments of the fat pad), where these structures are separated by a layer of muscle and an adipose tissue layer with a thickness greater than 1 mm. The length of the area with fat pad is always measured from the respective edge of the posterior wall.

These data were correlated with the left-right position of the esophagus, age, weight and sex of patients. We aimed to determine whether the LA morphology in the sagittal plane (the ratio of the height and depth, "roundness index") affects the nature of the contact of the LA posterior wall and esophagus. For further details of the measurements, please refer to Fig 1.

Statistical analysis

All data were tested using a normal distribution test at a significance level $\alpha = 0.05$. On the basis of the results of the tests for normal distribution, parametric analysis (a, b, d) or the non-parametric analysis (3c) was performed.

a) Analysis of the length of contact and length of fat pad above and below according to the "roundness index":

a statistical evaluation was performed using parametric correlation analysis (Pearson correlation coefficient).

b) Analysis of the length of contact and length of fat pad at the top and bottom according to the positions: a statistical evaluation was performed using analysis of variance (ANOVA) followed by post-hoc tests (LSD test and Tukey HSD test). All tests were performed at a significance level $\alpha = 0.05$.

c) The statistical significance of individual positions: a statistical evaluation was performed using a non-parametric sign test at a significance level $\alpha = 0.05$.

d) The width of the esophagus at various levels: a statistical evaluation was performed using a parametric t-test when the t-test was performed in relation to one reference constant diameter (significance level $\alpha = 0.05$).

RESULTS

Patient population

From September 2011 to August 2012, 56 consecutive patients who had undergone catheter ablation of atrial fibrillation were examined using multislice CT of the heart. Approximately three-quarters of the patients were males with a mean age of 60 years and a BMI that was slightly over 29. Most patients had no structural heart disease, had normal left ventricle function and had a slightly enlarged left atrium of an average diameter of 45 mm. Half of the patients were ablated for paroxysmal atrial fibrillation. For further details, please see Table 1.

The position of the esophagus relative to the left atrium

The esophagus was positioned most frequently in the left central part of the left atrium (column B, 26 pts., 46.4%) and the left lateral part of the left atrium (column A, 16 pts., 28.6%). In the middle of the left atrial posterior wall was 17.9% of the esophagi (column C, 10 pts.). The esophagus was found least often in the right middle and, specifically, in the right lateral part (column D, 3 pts., 5.4%, column E, 1 pts., 1.8% resp.). The statistically significantly least often position of the esophagus occurred on the right side behind the right-pulmonary veins ($P=0.001$). The most common esophageal position was behind the left side of the posterior wall of the left atrium in column B ($P=0.001$).

If we divide the position of the esophagus by the left atrium only at the position behind the left and right sides of the left atrium, then, similar to the study of Cury et al.⁸, the statistically most significant position of the esophagus is behind the left part of the left atrium (47 pts., 58.9% vs. 9 pts., 16%, $P=0.001$). In our group, we observed the esophagi reaching a maximum of two adjacent segments (Fig. 2).

Contact of the left atrium and esophagus

The average transverse diameter was 63.5 mm, the anteroposterior diameter was 48 mm, and the superoinferior diameter was 61 mm. The average width of the posterior wall was 56.1 mm. The average ratio of the anteroposterior diameter and superoinferior diameter, which is known

Table 1. Patient characteristics.

Patient characteristics	
Number of patients	56
Age	59.80 ± 9.59
Male	44 (78.57 %)
Ejection fraction of left ventricle	57.12 ± 7.95
Size of left atrium	45.20 ± 6.00
Body mass index	29.15 ± 4.33
Structural heart disease	6 (10.71 %)
Hypertension	27 (48.21 %)
Paroxysmal atrial fibrillation	29 (52.73 %)
Persistent atrial fibrillation	24 (43.64 %)
Long standing persistent atrial fibrillation	1 (1.82 %)
Atypical atrial flutter	1 (1.79 %)

Table 2. Width of the esophagus and distance of the esophagus-LA at each position.

Position of measurement	Average width of eso (mm)	Average distance eso - LA (mm)
1	15.23 ± 3.74	5.87 ± 2.44
2	15.70 ± 4.14	3.97 ± 1.29
3	15.89 ± 4.20	3.63 ± 0.95
4	16.45 ± 4.38	4.39 ± 1.30
5	16.56 ± 4.15	6.34 ± 2.12
Average 1 - 5	15.97 ± 4.12	4.84 ± 1.62
	$P \geq 0.130$	

eso - esophagus, LA - left atrium

Table 3. Correlation between the length of close contact and left-right distribution of positions of esophagus.

Position of esophagus	Number of cases (n)	Length of tight contact (mm)	Length of upper fat pad (mm)	Length of lower fat pad (mm)
A	16	50.5 ± 8.9	9.8 ± 5.8	16.8 ± 6.7
B	26	48.6 ± 11.4	9.5 ± 5.8	15.8 ± 7.3
C	10	50.4 ± 10.4	8.6 ± 4.4	17.0 ± 7.6
D	3	*68.9 ± 7.1	*2.4 ± 2.1	*12.2 ± 7.8
E	1	40.9	10.1	15.9
		$P=0.035$	$P=0.291$	$P=0.314$
Left	47	49.2 ± 10.4	9.6 ± 5.5	16.7 ± 7.3
Right	9	56.7 ± 12.5	6.7 ± 5.2	10.8 ± 5.6
		$P=0.060$	$P=0.153$	$P=0.027$

as the "roundness index of the atrium", was 1.3 (0.94 to 2.04, median 1.28).

The average width of the esophagus was 15.97 ± 4.12 mm, and there was no statistically significant difference between the widths of the esophagus at various levels ($P \geq 0.130$) (see Table 2). The average length of the posterior wall was 75.3 ± 8.9 mm. An average of 50.4 ± 11 mm of the posterior wall was in very close contact with the esophagus (median 51.2 mm, range from 25.7 to 76.6 mm). The average distance of the esophagus from the LA posterior wall was 4.8 ± 1.6 mm. The distance of the

esophagus and the LA was shorter in the central and superior part of the posterior wall; the esophagus is closest to the left atrium in plane No. 3 in the middle of the LA posterior wall, where the average distance was 3.63 ± 0.95 mm, while the most distant was in the inferior part of the posterior wall, where the average distance was 6.33 ± 2.11 mm. The measured values were statistically significantly different from the average value of 4.84 mm at each level ($P < 0.012$) (see Table 2).

The fat pad between the esophagus and the left atrium was observed in all patients, and it was discontinuous in 98.3% of patients. In the middle part of the LA PW, the muscle of the esophagus was adjacent directly to the muscle of the left atrium. In only one patient (1.7%), a thin layer of fat pad was observed throughout the whole contact of the esophagus and posterior wall of the left atrium with a thickness from 0.8 to 0.9 mm. For the rest of the patients, the fat pad constituted wedge-shaped segments between the superior and inferior parts of the esophagus and the left atrium (see Fig. 3). The average length of the fat pad on the superior and inferior parts of the LA were 9.1 ± 5.5 mm and 5.8 ± 7.3 mm, respectively. The superior segment of the fat pad was statistically significantly shorter ($P = 0.001$). There was no correlation between close contact length and the length of the segments of the fat pad in the superior and inferior parts ($r = 0.494$, $r = 0.482$, respectively).

Statistical analysis using correlation analysis did not reveal any effect of the shape of the LA in the sagittal plane, or "roundness index", on the contact of the esophagus with the posterior wall of the left atrium ($r = 0.106$). No statistically significant correlation was found between the length of close contact of the esophagus and left atrium, the length of the superior segment of the fat pad and inferior segment of the fat pad and age ($r = 0.116$, $r = 0.023$, $r = 0.263$), BMI ($r = 0.111$, $r = 0.065$, $r = 0.022$), and gender ($P = 0.976$, $P = 0.152$, $P = 0.865$).

Correlation of the length of close contact and length of the superior and inferior fat pads to the position of the esophagus towards the right atrium (the position behind the left atrium A - E and the position on the left or right) revealed statistically significant results. The length of close contact was significantly longer in column D (on average 68.9 ± 7.1 mm, $n = 3$, $P = 0.035$). This difference was also apparent when recalculated to the position of the esophagus on the left or right; however, it did not reach statistical significance (49.2 ± 10.4 mm on the left versus 56.7 ± 12.5 mm on the right, $P = 0.060$).

Longer area of close contact in column D, or behind the right part of the left atrium, was given by shorter length of the superior and inferior segments of the fat pad. However, only shortening of the lower segment of the fat pad reached statistical significance (for details, please see Table 3).

For examples of LA PW contact with the esophagus, please see Fig. 4.

Fig. 2. (A) Graphs of esophageal position frequency, (B, C) Examples of different positions of the esophagus in the postero-anterior view, (B) Extremely left lateral position A, (C) Right lateral position B

Fig. 3. Nature of fat pad. (A) Continuous fat pad extended throughout the entire length of contact of the left atrium and esophagus, green arrows, (B) Discontinuous fat pad, superior segment of fat pad is highlighted by yellow arrows, the inferior segment of the fat pad is highlighted by blue arrows, the area of direct contact of muscle of the esophagus with musculature of the left atrium is highlighted with red arrows.

DISCUSSION

Main findings

The position of the esophagus was significantly more often behind the left part of the left atrium, usually in position B. The average width of the esophagus was 15.97 mm, which was 28.6% of the width of the posterior wall of the left atrium. The esophagus was in very close contact with the posterior wall of the left atrium in the long section. According to our measurements, we obtained an average of 50.4 mm, which is an average of 66.9% of the length of the posterior wall.

Fig. 4. Examples of different types of contact of the esophagus and left atrium, side view, (A) Maximum contact, the area of close contact goes virtually from the roof of the left atrium up to 3/4 of posterior wall of left atrium, (B) Minimal contact, esophagus and left atrium are in contact in a very short section of the upper third of the posterior wall of the left atrium, (C) Displacement of the area of close contact caudal direction, a short area of close contact is in the lower third of the posterior wall, (D) Shift of the area of close contact in cranial direction, long area of close contact begins completely cranially at the superior edge of the posterior wall and extends up to middle of the posterior wall, (E) Nature of the contact of the esophagus and left atrium in a patient with a "round atrium" with a ratio anteroposterior/superoinferior diameter of 0.94. The area of close contact was significantly shifted in the cranial direction and occupied a greater part of the length of the posterior wall, (F) Nature of the contact esophagus-left atrium in a patient with "flat atrium" with ratio anteroposterior/superoinferior diameter 2.04; the area of close contact was relatively short and was placed at 3/4 of the length of the posterior wall.

The fat pad between the esophagus and the posterior wall of the left atrium was observed in all patients, but in 98.3% of patients, it was discontinuous and the esophagus was adjacent to the muscle of the left atrium in the middle part of the posterior wall of the left atrium. The smallest average distance of the lumen of the esophagus from the LA lumen was only 3.63 mm and was observed in the middle part of the LA PW. The area of close contact between the left atrium and esophagus was shifted upwards to the superior edge of the posterior wall of the left atrium. The area of close contact was significantly longer for the esophagi located behind the right side of the left atrium. We did not find any clinical factors predicting the position of the esophagus and esophageal contact with the left atrium.

Position of the esophagus relative to the left atrium

In accordance with previous works^{3,8-10,12,13}, the esophagus is significantly more often positioned behind the left part of the left atrium and is typically in position B. Most studies have determined the position of the esophagus relative to the left atrium using a different method: by measuring the distance of the esophagus from spacing between the pulmonary veins in a transverse section^{8-10,13}. However, these results are contradictory. For example, Cury et al.⁸ observed that the most common esophageal position was behind the left part of the LA. Maeda et al.¹³ divided the esophageal positions into A and B, respectively, A1, A2, B1 and B2, in the direction from the ostium of the left-sided pulmonary veins to the ostium of the right-sided pulmonary veins, where 74% of patients had an esophagus in group A1. Only Kottkamp et al.¹¹, who evaluated the position of the esophagus using a similar method as that described in our work, determined that the most common esophageal position was position C behind the middle part of the left atrium. Moreover, the vast majority of the other positions were in column A and B. The incidence of the esophagus behind the right side of the left atrium was generally minimal, ranging from 6% to 10% (ref.^{9,13}).

Contact of the left atrium, esophagus and fat pad

The esophagus in our cohort of patients is in very close contact with the posterior wall of the left atrium and is sectioned long (on average 50.4 mm). In this area, the esophagus is in close contact with the LA PW without the fat pad in 98.3% of patients from our cohort. Our values are within the upper limit of the range of the results of previous studies, in which the average length of close contact was within a broad range (from 26.2 to 58.0 mm, extreme values of 5.4 mm and 97 mm) (ref.^{9,12,10}). The new finding of our study is the asymmetry of areas with the fat pad. According to our measurements, the width of the superior segment is substantially shorter than the inferior segment (9.1 ± 5.5 vs. $15.8 \text{ mm} \pm 7.3 \text{ mm}$, $P=0.001$). These findings contrast with the work of Wang et al., where the length of the superior and inferior fat pads was similar ($14.1 \pm 7.2 \text{ mm}$ and $13.82 \pm 6.6 \text{ mm}$, respectively.)

In our cohort of patients, a shift in the area of close contact in the cranial direction to the superior edge of

the posterior wall is evident. This shift also corresponds to the average distance of the lumen of the esophagus from the lumen of the left atrium, which are smaller in the superior part of the left atrium (see Table 2). These results are inconsistent with those obtained by Wang et al., who split the posterior wall of the left atrium into four quadrants and found that the esophagus is most often in contact with the inferior quadrants, particularly with the left inferior quadrant (98% of esophagi is in contact with this quadrant). The cause of this discrepancy is still unclear. However, these results are difficult to compare due to the use of different measurement methodologies. Our results are consistent with those of Ho et al.³, such that anatomical preparations measured the thickness of the posterior LA wall within a range from 2.5 to 5.3 mm (mean, 4.1 ± 0.7 mm), and the distance between the endocardial surface of the left atrium and the esophageal wall is <5 mm in 40% of the 15 cadavers. Lemola et al.⁹ indicated that the average shortest distance of the LA lumen and the lumen of the esophagus was 3.5 ± 1.0 mm. The average width of the esophagus is 16 mm, which is within the range of the width of the esophagus in previously published studies (from 11 to 24 mm) (ref.^{8-10,12}).

Left-right distribution of close contact and prediction of contact from clinical factors

Another new element is the uneven distribution of the length of close contact, which is dependent on the left-right position of the esophagus to the left atrium. In our patients, we found a significantly longer area of close contact at the esophagi located behind the right side of the left atrium, particularly in column D. In these patients, close contact displacement in the cranial direction is maintained. The average length of the superior segment of the fat pad is only 2.4 mm. In this study, our results differ from those obtained in Wang et al.¹², which describes much more frequent contact of the esophagus with the inferior quadrants of the posterior left atrial wall, particularly with the left inferior quadrant (98% of the esophagi). However, the cause of this discrepancy is not clear. A limitation of our finding is the small number of patients with right-sided localization of the esophagus (column D, $n=3$, column E, $n=1$, right-sided localization $n=9$). If this finding could be confirmed in a larger cohort of patients, then it would be necessary to pay close attention during ablation in the vicinity of the right pulmonary veins along the entire length of the posterior wall of the left atrium.

Unfortunately, we could not define any clinical parameters (age, sex, BMI) predicting the nature of the contact of the esophagus and left atrium. The same conclusions were also obtained by Wang et al.¹², who observed the effect of gender on the width and length of the area of close contact, as well as Lemola et al.⁹ who failed to prove any correlation between age, sex, BMI, and the size of the left atrium as well as the presence and thickness of the fat pad.

The esophagus is a mobile structure in both the long-^{14,15} and short-term^{16,17}. A static image of the esophagus itself has limited value. However, clinical effect of

the adipose tissue (fat pad) between the esophagus and left atrium, which have been described in detail in our study and other studies, remains unclear. Moreover, we do not know whether this layer contributes to the protection of the esophagus prior to thermal damage during radiofrequency ablation. Thus, a longer area of physical contact between the esophagus and left atrium in the case of right-sided locations of the esophagus can be given by a small number of patients in this group, and it would require a larger group of patients to definitively confirm this conclusion.

CONCLUSION

From our results, it is apparent that the esophagus is a greatly variable structure in relation to the left atrium, both in regards to the distribution of the positions of the esophagus to the left atrium in the AP projection, as well as the contact of the esophagus with the posterior wall of the left atrium. The location of the esophagus in the posterior mediastinum is variable. Although the esophagus is most often located behind the left side of the left atrium, it may be situated in the entire area of the posterior wall of the left atrium and adjacent pulmonary veins. The area of close contact of the esophagus and posterior wall of the left atrium is relatively large, and it is on average $2/3$ of the length and $1/4$ of the width of the posterior wall of the left atrium and is shifted toward the left atrial roof. It begins just below the transition of the roof and posterior wall of the left atrium; in the area of posterior mitral annulus at the bottom part of the posterior wall, in most cases, there is a relatively large space filled with a fat pad.

Without imaging the esophagus using a specific imaging method, we were unable to predict where the esophagus was located in a particular patient during the procedure and the proximity of its contact with the posterior wall of the left atrium. The risk of damage to the esophagus during catheter radiofrequency ablation is in the area of the posterior wall of the left atrium and adjacent pulmonary veins and thus we have to take into account this fact and try to reduce that risk using all available methods.

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Antimüllerian hormone (AMH) levels and antral follicle count (AFC) as predictors of the ovarian reaction in young healthy women

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Objective. To determine the predictive capacity of antimüllerian hormone (AMH) and antral follicle count (AFC) on the ovarian reserve in a population of young women who are not treated with infertility.

Study design. A homogeneous collection of 61 individuals was subjected to testing by AMH, and to an ultrasound AFC test; and then was stimulated by the recombinant follicle stimulating hormone (FSH) in a dosage of 120 – 260 IU/L (mean $\pm$ standard deviation: 184.8 ± 36.36 IU/L) depending on the age and BMI.

Results. The number of metaphase II oocytes (MII) retrieved was 16.8 ± 8.38 counts; the total number of retrieved oocytes (OR), i.e. the number of germinal vesicle + metaphase I + metaphase II oocytes, was 21.6 ± 10.65 counts. Linear mixed models were used to verify effects of AMH and AFC on the ovarian reaction. The results showed that the mean number of MII oocytes significantly increases by both AMH (0.6-fold; 95% confidence interval (CI): 0.05, 1.24; $P=0.033$) and AFC (1.3-fold; CI: 0.24, 2.45; $P=0.018$). The mean of OR was significantly increased by AMH (1.0-fold; CI: 0.19, 1.73; $P=0.015$).

Conclusion. Both AFC and AMH are suitable predictors for the number of metaphase II oocytes. For predicting ovarian reserve is AMH sufficient.

Key words: AMH, AFC, ovarian reaction, ovarian reserve

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INTRODUCTION

When the follicle stimulating hormone (FSH) was introduced into the stimulation schemes, there has been an aim to avoid hyper-stimulation syndrome and the possible under-dosing of the FSH by predicting the ovarian response to stimulation. Different parameters such as age, weight, FSH, LH (luteinising hormone), AFC (antral follicle count), AMH (antimüllerian hormone), and challenge tests were tested to predict the ovarian reactivity. AMH glycoprotein is part of a group of transformation factors and in adult women it is produced by granulosa cells of antral and preantral follicles. The task of the AMH in the development of the female genital tract is described by Jost in 1947 (ref.¹). Lee in 1996 (ref.²) described the possibility to detect the levels of AMH in the serum of adult women. De Vet in 2002 (ref.³) defined the relationship between the lower levels of AMH and ovarian aging and in the same year Van Rooij (ref.⁴) reported the possibility to predict the reactivity of ovaries. These authors demonstrated a high correlation between the levels of AMH and ovarian reactivity, and a statistically significant difference between the AMH serum levels among the standard responsive population (more than 4 acquired oocytes/aspiration) and the population with a low ovarian response (poor responders). Fanchin⁵ found in the level of AMH a significantly higher correlation to the number

of gained oocytes than in inhibin B, oestradiol, FSH and LH. Another prospective study⁶ found significantly lower levels of AMH in the patients with a cancelled IVF (in vitro fertilisation) cycle in comparison with patients who finished IVF cycle by embryotransfer. The same author refers⁷ to AMH as a predictor with the strongest link to the number of gained oocytes in the stimulation, they recommended to evaluate the combination of the AMH, AFC and inhibin B levels to improve prediction of ovarian response. Meta-analysis in the work of Broekmans⁸ showed AMH and AFC as the most useful parameters for the prediction of ovarian response. The AMH has become a standard parameter examined by IVF patients and traditional FSH and LH were suppressed to the background.

In addition to the prediction of low ovarian reactivity, the AMH was also proposed as an indicator of ovarian damage in patients undergoing chemotherapy^{9,10}. Higher AMH levels are connected to higher risk of developing ovarian hyper-stimulation syndrome¹¹.

Tomás¹² refer for the first time to the AFC as a possibility to predict a poor or excessive ovarian response. Banci¹³ described a significantly higher predictive value of the AFC compared to baseline values of the FSH, inhibin B and estradiol. Follicles up to 10 mm were considered antral follicles. Fratarelli¹⁴ defined the value of 4 and less antral follicles as a limit for a significantly increased risk of cycle cancellation and connected to a decreased pregnancy rate.

This work is focused on the prediction ability of AMH levels, maternal age, FSH, body mass index (BMI) and AFC on the ovarian response, in the cohort of healthy women undergoing ovulation induction but not treated for infertility. The objective is to determine whether these parameters influence the total number of retrieved oocytes (OR) and the number of oocytes in the metaphase II stage (MII), and, so optimize the stimulation scheme for future. In our study FSH was used for stimulation of ovaries therefore FSH is considered as a random effect in a linear mixed model.

MATERIALS AND METHODS

The experimental cohort of 61 healthy women was chosen from women undergoing egg collection as donors of eggs, with an assumption of good fertility. Previous history of infertility was the reason for the exclusion from the study. Women were selected for the study according to a standard scheme: age 18–35 years (mean $\pm$ standard deviation: 23.9 ± 3.72 years), negative history of infertility, completely healthy without regular medication, infections negativity (HIV, syphilis, hepatitis B, C), genetic tests - normal karyotype (46 XX) without the presence of mosaics and translocation, negative examinations of 36 mutations of cystic fibrosis. The FSH dose (184.8 ± 36.36 IU/l) was determined according to the BMI (23.1 ± 3.41) and age. All of them were ultrasound examined to exclude clinically silent gynaecological pathology. All of them signed informed consent with participation in this study.

Stimulation with the recombinant FSH in the antagonistic protocol was triggered by the application of 0.2 mg agonist GnRH as a prevention of hyper-stimulation syndrome (OHSS). Egg collections were performed transvaginally by the Cook system under general anaesthesia.

Study design

The measurement of the AFC (10.5 ± 1.91 counts) was performed on the Medison Sonoace X6 ultrasound, the antral follicles visible by ultrasound up to the size of 8 mm were counted. The AMH (6.0 ± 3.52 ng/mL) was examined from venous blood and its level was measured with the paramagnetic particle chemiluminescent immunoassay method (Access AMH). The FSH dose was determined according to the BMI and age of individuals with respect to the following schedule: the individuals were divided into two groups by age (20–29 years, 30–34 years). Stimulation by the recombinant FSH was done in the dosage of 120–260 units daily according to the BMI: approximately 150 IU for BMI 22–25, 175 IU for BMI 26–28, 200 IU for BMI 28–30, 250–262 IU for BMI over 30 in short protocol with GnRH antagonist. More about basic anthropometric and biochemical characteristics of individuals is shown in Table 1.

Statistical analysis

The linear relationship among the studied factors AMH, FSH, BMI, OR, MII, AFC was investigated by

means of the correlation analysis (Pearson correlation coefficient). The effect of predictors on the total number of oocytes and the number of MII oocytes was analysed using linear mixed models. Each model consisted of fixed basic variables and one random effect. Continuous fixed variables were: AMH level, AFC and informative additive covariates as the age and BMI of the individual. Each model included FSH as a random effect. Statistical significance of fixed covariates was verified by means of F-test with between-within type adjustment of denominator degrees of freedom. Threshold of significance was defined at a *P* value of ≤ 0.05 . For random effect FSH standard variance structure was assumed in both models (uncorrelated FSH levels with homogeneity in variance). Restricted maximum likelihood (Reml) was used as estimation method for the variance-covariance parameters (variances of FSH levels and of random errors) in mixed models. SAS Version 9.4 (SAS Institute Inc., SAS Campus Drive, Cary, NC 27513, USA) was used for statistical analysis.

RESULTS

Results obtained from linear mixed models demonstrated a significant impact of the AMH level on the total number of oocytes retrieved (1.0-fold increase; CI: 0.19, 1.73; *P*=0.015) and the number of MII oocytes (0.6-fold increase; CI: 0.05, 1.24; *P*=0.033). The influence of the AFC was proved significantly only in relation to the number of MII oocytes (1.3-fold increase; CI: 0.24, 2.45; *P*=0.018). The influence of the AFC on the total number of oocytes retrieved was not proven; only a weak positive correlation was revealed among these two variables (*R*=0.28, CI: 0.03, 0.50; *P*=0.028). The relationship between AFC and the number of MII oocytes was also only weak and positive (*R*=0.35, CI: 0.10, 0.55; *P*=0.006). From the other factors, the weak positive correlation was determined between AMH and the total number of oocytes (*R*=0.33, CI: 0.08, 0.53; *P*=0.011), and AMH and the number of MII oocytes (*R*=0.30, CI: 0.05, 0.51; *P*=0.021). In the given age range, only insignificant link between the age and the number of retrieved oocytes was found; the BMI did not emerge as significant in either of the parameters. Among the groups of individuals (formed according to age and BMI), no differences were found in the number of MII oocytes and the total number of oocytes retrieved; the random FSH dose factor therefore has no statistically significant effect on the total number of oocytes nor the number of MII oocytes. For details see Table 2 and 3.

DISCUSSION

Similar conclusions as those of ours, i.e. the positive correlation between the AFC and the number of retrieved eggs by donors, are described by Vrontikis¹⁵; these authors also found a link between the AFC with continuing pregnancies among recipients. Riggs¹⁶ correlated the AMH and ovarian response to stimulation by egg donors

Table 1. Basic anthropometric and biochemical characteristics of individuals in the cohort.

Variable	Mean	Standard deviation	Lower quartile	Upper quartile
MII oocytes [counts]	16.8	8.38	11.0	22.0
OR [counts]	21.6	10.65	15.0	28.0
AMH [ng/mL]	6.0	3.52	3.5	7.9
AFC [counts]	10.5	1.91	10.0	11.0
BMI	23.3	3.72	20.6	25.0
Age [years]	23.9	3.72	21.0	27.0
FSH [IU/L]	184.8	36.36	150.0	225.0

Table 2. The results of the linear mixed model for the number of MII oocytes response. The symbol DF means the number of degrees of freedom of F-test with between-within type adjustment of denominator degrees of freedom, the value of F-test is denoted as F. The last two columns denote the lower and upper bound of 95% confidence interval for an effects.

Effect	Estimate	Standard Error	DF	F	P value	Lower CI	Upper CI
AMH	0.65	0.30	56	4.77	0.033	0.05	1.24
AFC	1.34	0.55	56	5.93	0.018	0.24	2.45
BMI	0.37	0.28	56	1.74	0.192	-0.19	0.93
AGE	-0.23	0.33	56	0.49	0.487	-0.90	0.44

Table 3. The results of the linear mixed models for the total number oocytes (OR) response. The symbol DF means the number of degrees of freedom of F-test with between-within type adjustment of denominator degrees of freedom, the value of F-test is denoted as F. The last two columns denote the lower and upper bound of 95% confidence interval for an effects.

Effect	Estimate	Standard Error	DF	F	P value	Lower CI	Upper CI
AMH	0.96	0.38	56	6.27	0.015	0.19	1.73
AFC	1.10	0.71	56	2.41	0.127	-0.32	2.53
BMI	0.42	0.36	56	1.32	0.256	-0.31	1.15
AGE	-0.12	0.41	56	0.09	0.767	-0.95	0.70

- correlation with the quality of embryos and ongoing pregnancy has not been established; in addition, the meta-analysis performed by Reshef¹⁷ showed only a weak correlation between the AMH and the clinical pregnancy rate. Reijnders¹⁸ studied the link between the results of IVF pregnancy cycles and the level of AMH by women with an extremely low ovarian reserve; in the group with a higher AMH they demonstrated a better prognosis in terms of pregnancy. However, their results also included patients who did not have the embryo transfer (oocyte was not retrieved or the embryo stopped in its development); it is therefore rather the effect of a small number of oocytes. Nelson et al.^{19,20} found a significantly stronger link between the level of AMH and response to FSH stimulation compared with the AFC. They found positive monotonic relationship between the AMH and number of oocyte retrieved. Spearman correlation coefficient for AMH was 0.56 (CI:0.50-0.62) for agonist trial and 0.55 (CI:0.50-0.60) for antagonist trial which was stronger than for AFC (agonist 0.28, CI:0.20-0.36 and antagonist 0.33, CI:0.26-0.39). Our results are based on Pearson correlation coefficient so the linear relationship is verified. Further in Nelsons retrospective multi-centre study AMH was identified as the most important factor for the prediction of the total number of oocytes and inclusion of AFC into the model has no significant improvement influence.

So we obtained the same results in our study, moreover, we evaluated the impact of the AMH level on the total number of oocytes retrieved (1.0-fold increase; CI: 0.19, 1.73; $P=0.015$). The improvement of our study is verification that both AMH (0.6-fold increase; CI: 0.05, 1.24; $P=0.033$) and AFC (1.3-fold increase; CI: 0.24, 2.45; $P=0.018$) are useful factors for predicting the number of MII oocytes. Pin-Yao et al.²¹ observed among young women under 35 years of age a correlation between the AMH and number of retrieved oocytes, and pregnancy outcomes of the IVF cycles. Low AMH was correlated with a lower total number of oocytes, number of MII oocytes and also with higher probability of the IVF cycle cancellation. Nevertheless, the pregnancy rate for embryo transfer as well as the delivery rate was the same in both groups. From the above, it is evident that both the AMH and AFC are especially indicators of the anticipated number of retrieved oocytes; it has not been proven their usefulness as a predictor of the oocytes quality, embryo quality and pregnancy rate. Sahmay et al.²² reported significant correlation between AMH level and a higher pregnancy rate by women over 35 years of age. That is again linked more probably with the higher number of retrieved oocytes by the FSH stimulation.

CONCLUSION

Both AMH and AFC proved suitable parameters for predicting ovarian response to FSH stimulation. AFC together with AMH are useful factors for predicting the number of MII oocytes; the total ovarian reserve (OR) can be predicted by AMH. The main advantage of AFC is low cost, because it can be examined during standard ultrasound examination. We recommend including both parameters in the standard examination schemes before determining the optimal dose of the FSH preparation.

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Meditation's impact on cognitive functions in mild cognitive impairment: A pilot study

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Background. Effect of meditation on various domains of cognition in aging and patients at risk of dementia is receiving growing attention. The potential of mindfulness to reduce, slow down or prevent cognitive decline in patients with high risk of developing dementia is a curious topic of discussion with vast clinical implications. However, the effect of Mindfulness-Based Interventions (MBIs) on cognitive functions in patients with cognitive decline is very poorly understood.

Aim. The aim of this study was to examine effects of Mindfulness-Based Stress Reduction (MBSR) program on cognitive functions in patients with mild cognitive impairment (MCI).

Methods. 14 MCI patients participated in the program. The severity of their cognitive decline was assessed by CogState cognitive tests.

Results. Results showed that, when comparing values before and after completing MBSR, MCI patients significantly improved in cognitive task assessing psychomotor functions, but not in tasks assessing attention, visual learning, or working memory.

Conclusion. Data suggest that mindfulness may positively influence certain cognitive domains in MCI patients. However, further studies with larger sample size, follow-up data and active control group are needed.

Key words: mindfulness, meditation, cognition, MCI, neurodegeneration, Mindfulness-Based Stress Reduction

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INTRODUCTION

Prevalence of dementia increases every year due to the increasing age of the world's population. It has been predicted that number of people with dementia may double every 20 years¹. Most common cause to dementia are neurodegenerative diseases, especially Alzheimer's disease (AD) (ref.²). Considering that there is no current cure for this disease, multiple studies are searching for preventative strategies that could effectively delay its onset. It has been stated that such prevention may even be more effective than current pharmacological treatment^{3,4}. Mild Cognitive Impairment (MCI), the symptomatic pre-dementia stage, has been an object of multiple trials in hope finding an effective therapy to slow down, or even prevent the progression to AD (ref.⁵).

There is a growing interest in studying effects of meditation as a potential strategy for preventing cognitive decline^{6,7}. Interventions using meditation techniques based on the concept of mindfulness have been the mostly researched⁷. Mindfulness refers to awareness emerging through paying attention, purposely, in the present moment, and without judgement to the unfolding of experience moment by moment⁸. Systematic review discussing the topic of effect of mindfulness-based interventions (MBIs) on cognitive functions showed that MBIs might influence various domains of cognition, such as attention, memory and executive functions⁹. There is, however, very limited number of studies assessing the effect of MBIs

on cognition in MCI (ref.¹⁰). Data from one study examining 7 MCI patients have suggested trend towards improvement of cognition¹¹, assessed by Alzheimer's Disease Assessment Scale cognitive subscale. This subscale, widely used in AD, has, however, been reported to be less responsive to change when used in patients with MCI (ref.¹²). The same study has also reported increased functional connectivity between the left hippocampus, posterior cingulate cortex and bilateral medial prefrontal cortex and trends of less hippocampal volume atrophy when compared to control participants. Authors concluded that MBSR may reduce hippocampal atrophy and improve functional connectivity in the brain areas also mostly affected by AD. It has also been concluded that further studies with larger sample size are needed¹³.

The aim of this prospective study was to examine effects of MBSR on cognitive functions in MCI and to expand our knowledge about which cognitive domains are mostly influenced.

METHODS

Patients diagnosed with MCI were selected from the Czech Brain Aging Study (for details, see table 1), epidemiological study based on longitudinal follow up of non-demented older subjects with cognitive complaints. 109 patients with MCI were invited over the phone to participate in this study. 14 participants accepted the invitation.

The sample consisted of 9 men and 5 women, aged 65-85 years ($M = 74.36$, $SD = 7.24$).

Participants were tested twice, immediately prior to commencement of the 8-week MBSR course and then again 2-3 days after its completion. Four computer CogState cognitive tests were administered assessing: attention (Identification Test), psychomotor functions (Detection Test), visual learning (One Card Learning Test), working memory (One Back Test) (ref.¹⁴). CogState tests are designed for repeated administration with minimal practice or learning effects, so it was possible to use them repeatedly in 8 weeks' distance.

Table 1. Study sample.

Participants	
n	13
Gender	8 male, 5 female
	Average $\pm$ Standard deviation
Age	74.69 $\pm$ 7.42
Years of education	14.38 $\pm$ 3.15

Statistical analysis

One patient was further eliminated as an outlier. Statistical analysis was therefore conducted on 13 patients. To compare pre-intervention and post-intervention values within the group, Wilcoxon test was used. Statistical analysis was performed using R and RStudio and the chosen significance level was $\alpha = 0.05$.

RESULTS

Preliminary data analysis (see Table 2.) showed that comparison of pre-intervention and post-intervention values within the group revealed that MCI patients showed significant improvement of CogState Detection Test (observed P-value 0.0479). Values from CogState Identification Test, One Card Learning Test and One Back Test did not show any significant differences before and after the therapy.

Table 2. Results.

Task	<i>P</i>
Cogstate DET	0.0479
Cogstate IDN	0.3396
Cogstate OCL	0.6355
Cogstate OBT	0.8241

DET - Detection Test, IDN - Identification Test, OCL - One Card Learning Test, OBT - One Back Test

DISCUSSION

This study linked up to previous research¹¹ by assessing a curious, but insufficiently researched topic of impact of mindfulness on cognitive functions in MCI. By almost doubling the sample size in comparison to previous re-

search¹¹ and by measuring various domains of cognition we were able to deepen our insight into possible impact of meditation on cognition in elders suffering from cognitive decline. Data suggest that participation in MBSR might be related to an improvement of psychomotor functions, but not in other domains, such as attention, visual learning, or working memory. Previous research on MCI have showed trend towards improvement in cognition¹¹ in task which primarily assesses global cognition in response to antedementia therapies¹². By using cognitive tests which have been previously defined as useful for identifying memory impairment related to MCI and no learning effect¹⁵, we have been able to assess a topic of mindfulness's impact on cognition more thoroughly. Our data suggest that mindfulness might influence certain aspects of cognition in MCI and might therefore be relevant when considering non-pharmacological approach to cognitive decline in this population.

These preliminary results stimulate need for future research of this topic in order to better understand the impact and relevance of meditation for MCI. Major limitation when interpreting these preliminary data is a current lack of data from an active control group, which makes it difficult to estimate the difference between test-retest effect, placebo effect and effect of MBSR intervention. There is a need for further studies, that would use larger sample size, compare results with an active control group and to make follow-up measurements later after the intervention has ended. Another topic curious topic to be researched is how to effectively deliver MBI with respect to particular characteristics of this population - manuscript assessing data related to this topic has already been submitted for review. Even though an adapted mindfulness intervention for patients suffering from dementia has been found feasible in past, authors declared that before adapted implementation is widely recommended, there is a need for more research to further assess its effectiveness¹⁶.

CONCLUSION

This pilot study found that elders with MCI completing MBSR may improve in cognition, particularly in psychomotor functions. Despite limitations of these preliminary results, such as small sample size, non-comparison to active control group, and current lack of follow-up data, this study is a next step towards revealing the question of impact of MBI on cognitive functions in those, who are already diagnosed with onset of cognitive decline.

Ethical approval

This study has been approved by ethics committee of St. Anne's University Hospital. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Auhor contribution: RS: manuscript writing, data interpretation, mindfulness state assessment; KS: creating study protocol, data interpretation, manuscript writing; RM: data interpretation, neuropsychological examination, manuscript writing; AJ: teaching MBSR intervention; MJ: data analysis, tables; JH: data interpretation, management of research group.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

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