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Short Reports

MEASURING MICRORNAS: COMPARISONS OF MICROARRAY AND QUANTITATIVE PCR MEASUREMENTS, AND OF DIFFERENT TOTAL RNA PREP METHODS

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Background: Determining the expression levels of microRNAs (miRNAs) is of great interest to researchers in many areas of biology, given the significant roles these molecules play in cellular regulation. Two common methods for measuring miRNAs in a total RNA sample are microarrays and quantitative RT-PCR (qPCR). To understand the results of studies that use these two different techniques to measure miRNAs, it is important to understand how well the results of these two analysis methods correlate. Since both methods use total RNA as a starting material, it is also critical to understand how measurement of miRNAs might be affected by the particular method of total RNA preparation used.
Results: We measured the expression of 470 human miRNAs in nine human tissues using Agilent microarrays, and compared these results to qPCR profiles of 61 miRNAs in the same tissues. Most expressed miRNAs (53/60) correlated well (R > 0.9) between the two methods. Using spiked-in synthetic miRNAs, we further examined the two miRNAs with the lowest correlations, and found the differences cannot be attributed to differential sensitivity of the two methods. We also tested three widely-used total RNA sample prep methods using miRNA microarrays. We found that while almost all miRNA levels correspond between the three methods, there were a few miRNAs whose levels consistently differed between the different prep techniques when measured by microarray analysis. These differences were corroborated by qPCR measurements.
Conclusion: The correlations between Agilent miRNA microarray results and qPCR results are generally excellent, as are the correlations between different total RNA prep methods. However, there are a few miRNAs whose levels do not correlate between the microarray and qPCR measurements, or between different sample prep methods. Researchers should therefore take care when comparing results obtained using different analysis or sample preparation methods.

Background

MicroRNAs (miRNAs) are small (~18–24 nucleotides) non-coding RNAs which bind to mRNAs to regulate protein expression, either by blocking translation and/or by promoting degradation of the mRNA target (reviewed in [1-3]), or alternatively by increasing translation [4,5]. They have been found to be involved in numerous functions such as cell fate determination, cell proliferation, cell differentiation, and cell death (reviewed in [6,7]). Profiles of miRNAs in various types of tumors have been shown to contain potential diagnostic and prognostic information (reviewed in [8,9]). The number of known miRNAs has rapidly increased in recent years, and currently there are 722 human miRNA sequences reported in the Sanger Institute's miRNA database release 10.0 (miRBase) [10-12], with potentially many more yet to be reported [13,14].

Several methods for global miRNA profiling are currently in common use. These include quantitative RT-PCR (qPCR) involving stem-loop RT primers combined with TaqMan PCR (Applied Biosystems) analysis [15,16], qPCR with locked nucleic acid primers (Exiqon) [17], qPCR using poly(A) tailing (QIAGEN, Stratagene) [18,19], high-throughput sequencing of small RNA libraries [20], and microarray analysis (for examples, see [2126]). Typical experimental workflows often involve using different methods of measuring miRNAs at different research stages. For this reason, it is important to know how well the different measurements agree with each other. Several groups have compared microarray profiling results with those obtained by quantitative PCR for either a small number of genes or a small set of samples [21,22,26-30]; however there has been no systematic comparison of larger numbers of miRNAs across a widely diverse range of human tissues using the two methods.

In this study, we compared the relative expression of 61 different miRNAs across nine different human tissues, measured using both Agilent miRNA microarrays and TaqMan qPCR. The Agilent microarray platform features the direct end-labeling and pro-

filing of mature miRNAs from total RNA without any size fractionation or amplification to minimize experimental loss, bias, or variations [31,32]. The labeling reaction is performed under denaturing conditions to provide high labeling yield, minimal sequence bias [26], and consistently reproducible efficiency for every miRNA sequence [31,32]. By incorporating hairpin structures in the microarray probe, basepairing with the additional nucleotide incorporated during labeling, and empirical melting point-determination, the platform is capable of single-nucleotide discrimination in the miRNA sequences while specifically distinguishing the mature miRNAs from longer RNAs in the total RNA sample [26,31,32]. We chose to compare this microarray system against the Taqman qPCR system in particular, since at the time this work was performed this was the most commonly utilized miRNA qPCR system. We found excellent correlation between the microarray and PCR results for most of the miRNAs. We further examined two of the miRNAs showing low correlations by using spiked-in synthetic RNAs, and found that differential sensitivity between the two techniques is not the cause of the discrepancy.

Another factor which could potentially affect the results of an miRNA profiling study is the method used to isolate RNA from the biological sample. Both the Agilent microarray system and the TaqMan qPCR systems use total RNA as the starting material; however, it is unclear whether different total RNA preparation methods will yield systematically different miRNA profiling results. In this report, we compared the results of miRNA microarray profiling obtained with three different commonly used total RNA prep methods. We found that the results for most miRNAs were equivalent among the different sample preparation methods, but that measured levels of a small number of miRNAs differed systematically.

Results and discussion

Quantitative RT-PCR and Agilent microarray miRNA profiles correlate strongly

We previously reported that a comparison of Agilent microarray profiling and SYBR green-based quantitative RT-PCR (qPCR) of ten miRNAs in seven different human tissues found the two measurements correlated quite well [26]. To perform a more extensive comparison, we analyzed the expression of 61 human miRNAs in nine different tissues (brain, breast, heart, liver, placenta, testes, ovary, skeletal muscle, thymus), using both Agilent miRNA microarrays and TaqMan stem-loop qRT-PCR [15]. Aliquots of the same RNA samples were used for both the microarray and qPCR measurements. We chose these particular 61 miRNAs for several reasons. First, they represent a wide range of expression levels, as determined in an initial array analysis of some of the tissues. Second, they have wide differences in GC content, ranging from 23% (miR-190) to 68% (miR-328). Third, we chose several miRNAs which had potentially problematic sequences or exhibited atypical behavior during the development of the Agilent microarray platform: two of these did not show as good a linear titration curve as other miRNAs tested in a previous study (miR-126*, miR-296) [26], and two other miRNAs were previously reported not to be labeled by enzymatic methods similar (but not identical) to that used with the Agilent microarray assay (miR-208, miR-219) [33].

Of the 61 miRNAs examined, only miR-637 was not detected by either method in any of the tissues. The rest of the miRNAs assayed were detected in most or all of the tissues by both methods, with two exceptions: miR-208, expressed only in the heart [34] and at very low levels in skeletal muscle, and miR-138, expressed in the brain, and at lower levels in placenta and thymus (all data is shown in Additional File 1).

The qPCR and microarray results were compared by plotting the qPCR cycle threshold (Ct) value versus the $\log_2$ of the array

signal for each miRNA in all nine tissues (representative plots are shown in Figure 1, with the remaining plots shown in Additional Files 2, 3, 4, 5, 6, 7). These two values should be directly comparable, since both the qPCR Ct value and the $\log_2$ of the microarray signal change by a value of 1 for every 2-fold change in miRNA concentration. If the qPCR and microarray measurements are equivalent, the plots will show a linear correlation (R = -1) with a slope of - 1. Figure 2 shows the slopes and the correlation values for each of the 60 miRNAs. 56 of the 60 miRNAs show correlation values (R) between - 0.8 and 1.0, and 50/60 plots have slopes between - 1.2 and - 0.8. Of the four miRNAs which were selected as potentially problematic in the microarray measurements, only miR-296 did not correlate between the microarray and qPCR assays; miR-208, miR-219, and miR-126* all gave excellent correlations.

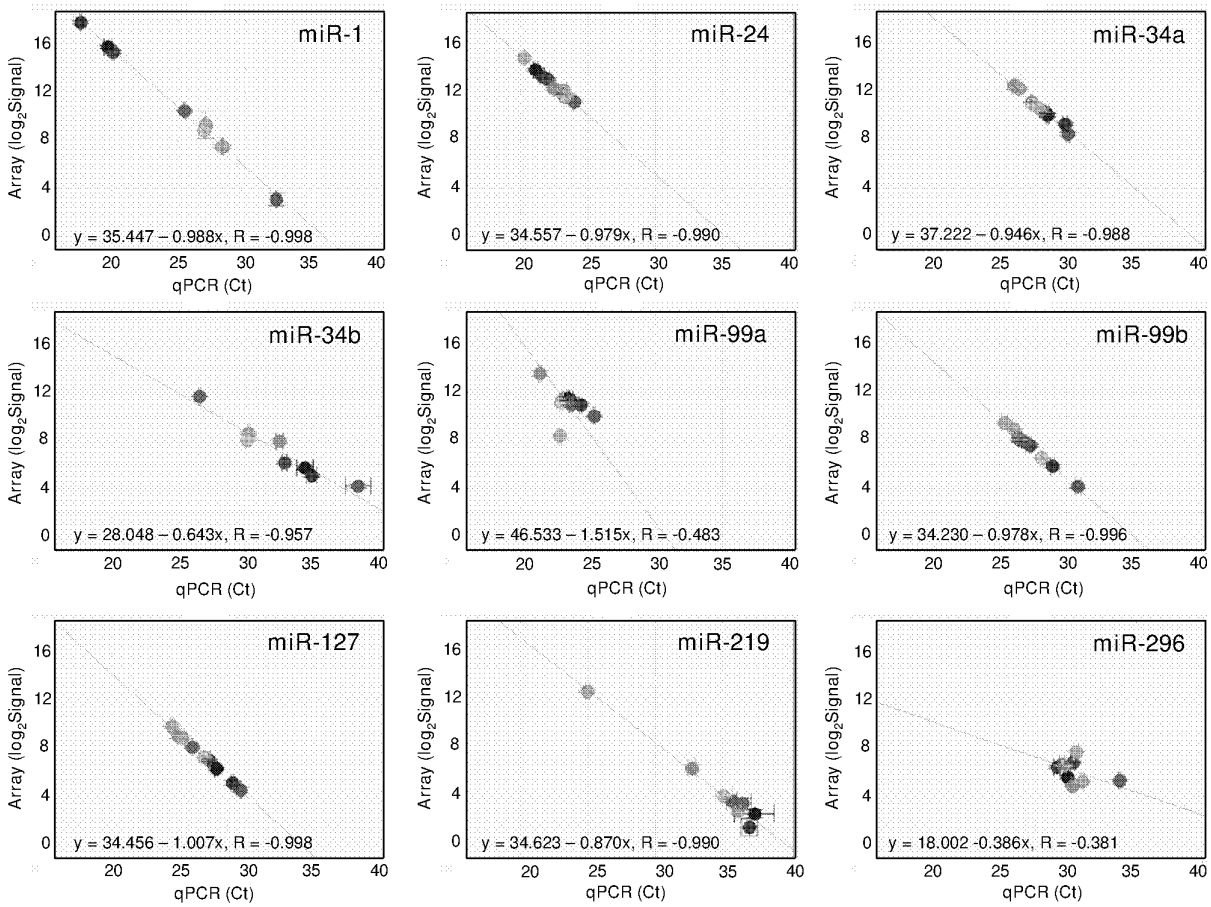


Figure 1. Comparison of qPCR and microarray miRNA profiling for individual miRNAs in nine human tissues. Scatter plots are shown for 9 of the 61 miRNAs assayed, with qPCR results (cycle threshold (Ct) values) on the x axes and microarray results ($\log_2$ of the total gene signal) on the y axes. Each data point represents one tissue. All plots are drawn to the same scale. The equations and R values on each plot are for the orthogonally-fitted line. Spot colors indicate the tissue: red = breast, pink = testes, dark blue = heart, light blue = placenta, dark green = liver, light green = ovary, orange = brain, brown = skeletal muscle, and grey = thymus. Tissues where qPCR results were flagged as "undetermined" by ABI software, or where log2 of the total gene signal on arrays was < 1, were not plotted. Error bars indicate standard deviation (SD) of Ct values for qPCR results and (SD/Mean)*$\log_2$e of the signals for the array results. Scatter plots for the remaining 51 miRNAs (one miRNA gave no signals with either qPCR or arrays) are in Additional Files 2, 3, 4, 5, 6, 7.

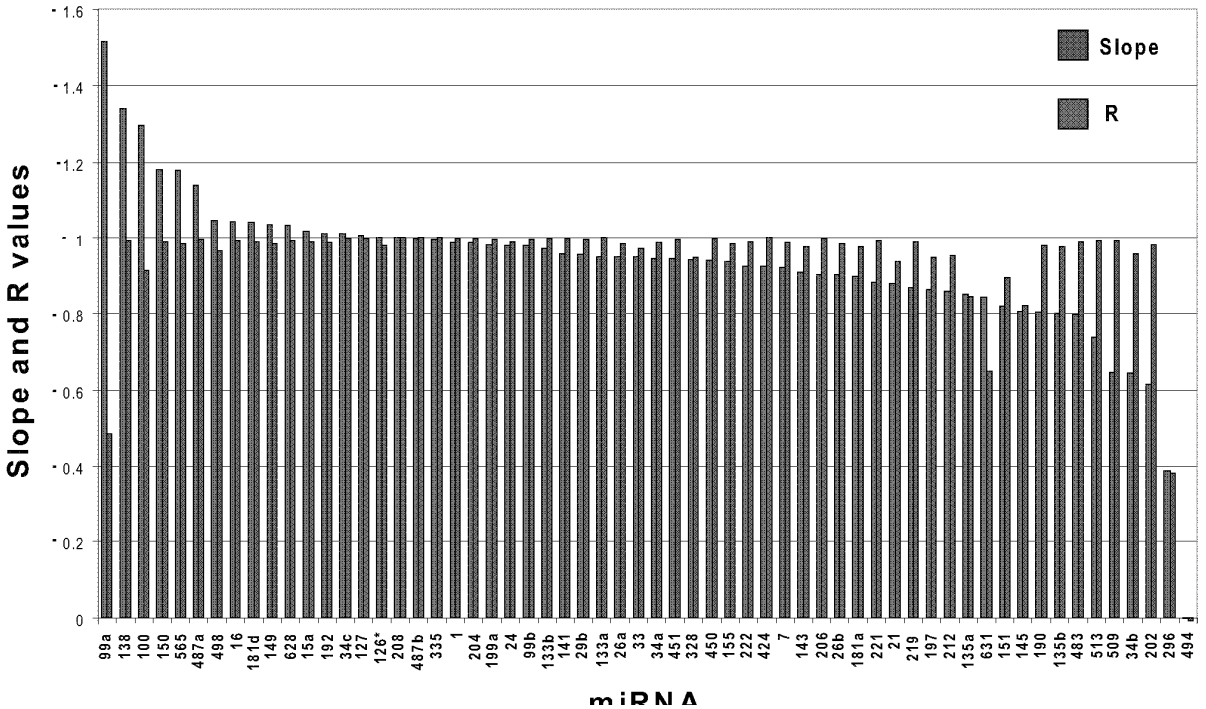


Figure 2. Slope and R values for all 60 miRNA scatter plots. The slopes and R values for the orthogonal fit lines for all 60 of the miRNAs plotted in Figure 1 and Additional Files 2, 3, 4, 5, 6, 7 are ordered by the slope values. Slopes are shown in blue and R values are in red.

To examine the results for all 60 miRNAs on one plot, we cannot simply plot qPCR Ct values versus microarray signals for all miRNAs in all tissues, because both the qPCR and microarray assays have differential sensitivities to different miRNAs. Thus, instead of looking at absolute expression levels, we must look at relative ratios of miRNA expression between two different tissues. To judge the consistency of fold-changes measured by microarray and qPCR platforms, we plotted the ratios of miRNA expression between all 36 possible pairs of tissues as measured by qPCR (Ct(tissue1)-Ct(tissue2)) and by microarrays (log2(signal in tissue1)-log2(signal in tissue2)). Four such plots are shown in Figure 3 (the other 32 plots are shown in Additional Files 8, 9, 10, 11, 12, 13, 14, 15), while Figure 4 shows the slopes and R values for each of the plots for the 36 tissue pairs. The plots all show very good correlation between the qPCR and array ratios, with R values between - 0.984 and - 0.821. The slopes of the 36 plots vary between - 1.05 and - 0.793. The intercepts of these fold-change plots (shown in Figures 3 and Additional Files 8, 9, 10, 11, 12, 13, 14, 15) indicate the consistency between fold-changes measured by the two methods. The mean of the intercepts of the line fits for the 36 tissue pairs was 0.00 +/0.23 (1 SD) (data not shown).

This level of variability (17%) is comparable to that seen among independent measurements using the same technique.

Measurements of spike-ins of miRNAs which systematically differ between platforms show linear sensitivity

miR-494, miR-296, and miR-99a are the three miRNAs that exhibit the most discrepant correlation values and slopes between the qPCR and microarray assays (Fig. 2); however, if the measurement of miR-99a in placenta is omitted, the slope for this miRNA becomes - 0.844 with R = - 0.929 (see plot in Fig. 1). For miR-494 and miR-296, if one platform were measuring levels of these miRNAs accurately, while the other

platform were not, then we might expect a significant divergence from linearity to be observed between the two measurements when adding increasing amounts of synthetic miR-494 or miR-296 RNA into a total RNA sample. To test this, we added 1 zmol to 10 fmols of synthetic miR-296 and miR-494 RNAs to 100 ng of total RNA from liver or placenta, and measured the qPCR and microarray responses (Figure 5).

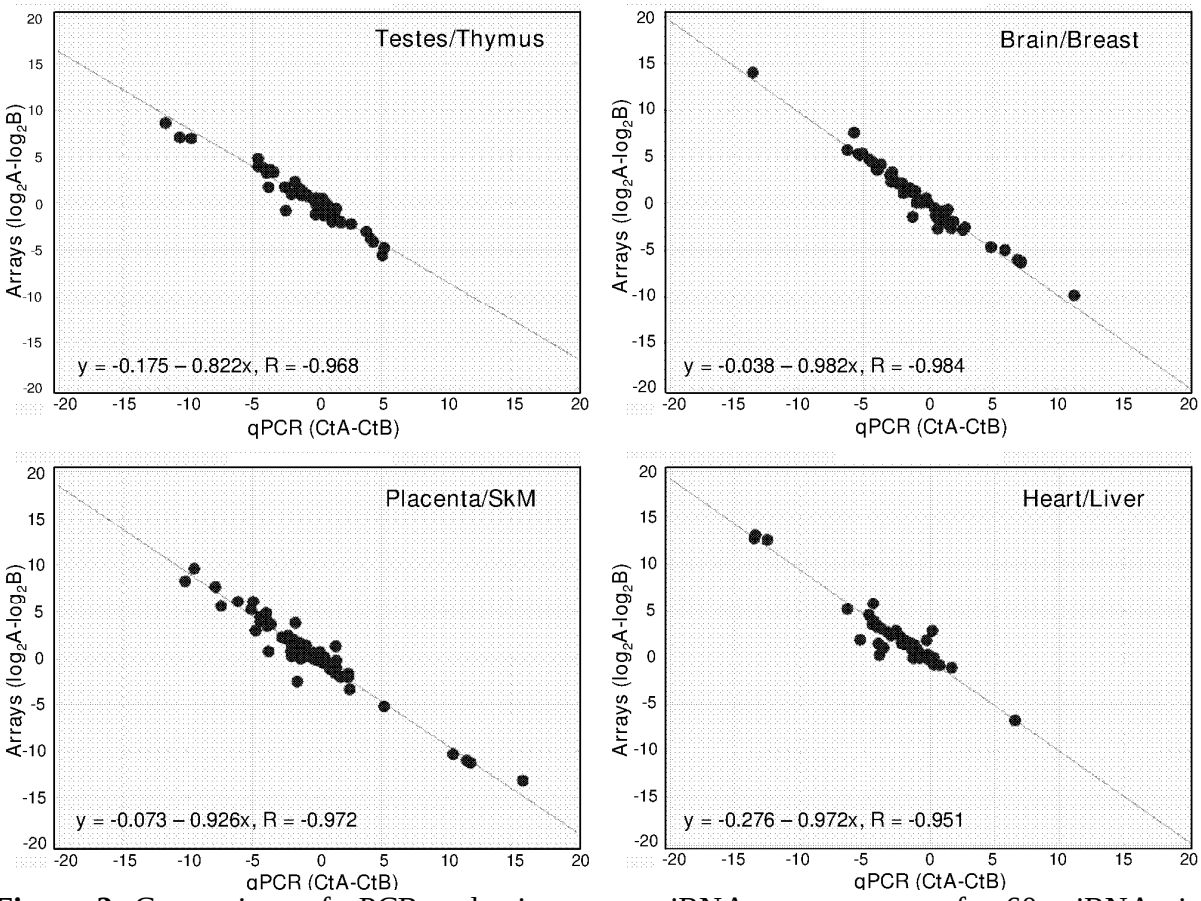


Figure 3. Comparison of qPCR and microarray miRNA measurements for 60 miRNAs in four tissue pairs. Scatter plots are shown for miRNA expression ratios in four different tissue pairs, as determined by qPCR (x axis) and microarrays (y axis), where each data point represents one miRNA. The qPCR values are the difference between the Ct values from the two tissues, and the microarray values are the difference between the $\log_2$(total gene signals) from the two tissues. The equations and R values on each plot are for the orthogonally-fitted line. Scatter plots for the remaining 32 tissue pairs are shown in Additional Files 8, 9, 10, 11, 12, 13, 14, 15. SkM = Skeletal Muscle.

For both miRNAs, in both tissues, the relation between qPCR measurement and array measurement is linear above a threshold spike-in concentration. The R values of the linear regions are very close to - 1, with slopes between - 0.842 and - 0.935, indicating that both the qPCR and the microarrays are producing sample-responsive and internally consistent measurements of miR-296 and miR-494 at these concentration levels. Below the threshold spike-in levels, the qPCR Ct values and microarray signals are unchanged for miR-494, while for miR-296 the Ct values increase slightly, but the array measurements are unchanged. We conclude that the difference between the two platforms is not due to different sensitivity, since both the microarray and qPCR measurements are

capable of measuring miR-296 and miR-494 accurately above a spike-in concentration threshold. Presumably some type of interference confounds the measurement of endogenous expression levels in the complex sample, on one or both of the platforms.

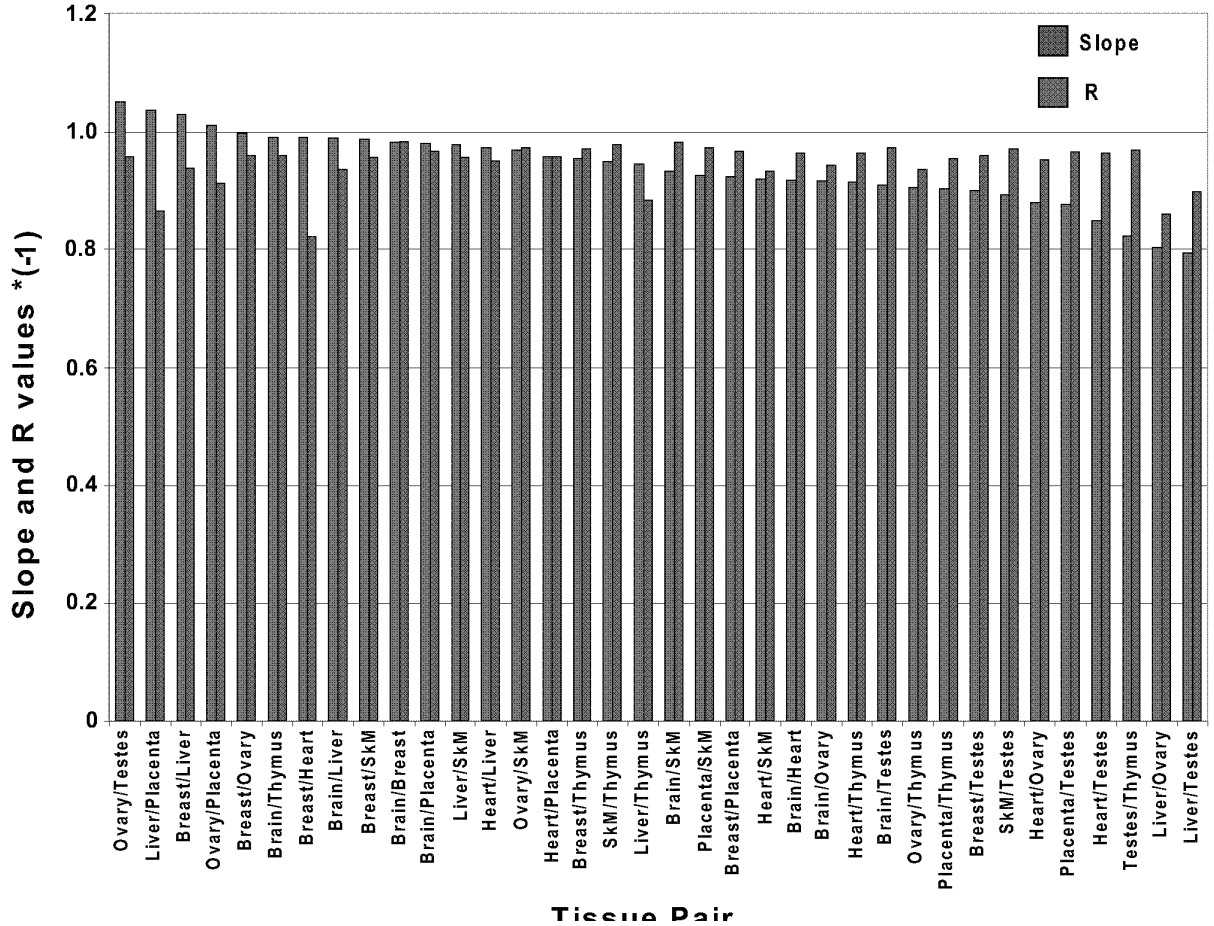


Figure 4. Slope and R values for all 36 tissue pair plots. The slopes and R values for the orthogonal fit lines for all 36 possible tissue pairs plotted in Figure 3 and Additional Files 8, 9, 10, 11, 12, 13, 14, 15 are ordered by the slope values. Slopes are shown in blue and R values are in red.

Three different total RNA preparation methods show similar yields and quality

A question which arises when comparing the miRNA profiling results reported in different studies is whether the methods used to isolate RNA from tissue or cell line samples systematically affect the miRNA profiles. To examine whether the miRNA profile of a sample is affected by the type of total RNA prep method used, we prepared one large frozen cell pellet from each of two different human cell lines, HeLa (a cervical carcinoma line) and ZR-75-1 (a breast carcinoma line). We then subdivided these pellets into equal aliquots, and performed total RNA isolation on the aliquots using three different techniques: phenol/guanidinium (TRIzol, Invitrogen) followed by isopropanol precipitation, and two column-based techniques, miRNeasy (QIAGEN) and *mir*Vana (ABI). Four to eleven replicate preps were performed on each cell type with each method.

Mean RNA yields, as measured by absorbance at 260 nm, and quality metrics for each prep type are shown in Table 1. The RNA integrities of the preps were analyzed on the Agilent 2100 Bioanalyzer, and all the preps had high quality RNA according to the

RIN number [35,36]. This indicates that most of the RNAs in the various preps were intact, with minimal breakdown. However, RIN values do not provide information about non-RNA contaminants, such as organic reagents and DNA. The TRIzol preps showed the lowest mean 260/230 ratios, possibly indicating the presence of some remaining TRIzol reagent in the final product.

Since the absorbance at 260 nm is used to quantitate the amount of RNA for use in the measurement assays, and since the 260:230 ratios can only serve as a crude guideline to possible contaminants, it is important to examine the absorption spectra in more detail (Figure 6). Some of the spectra clearly show the presence of additional peaks between 220–230 nm, indicating the

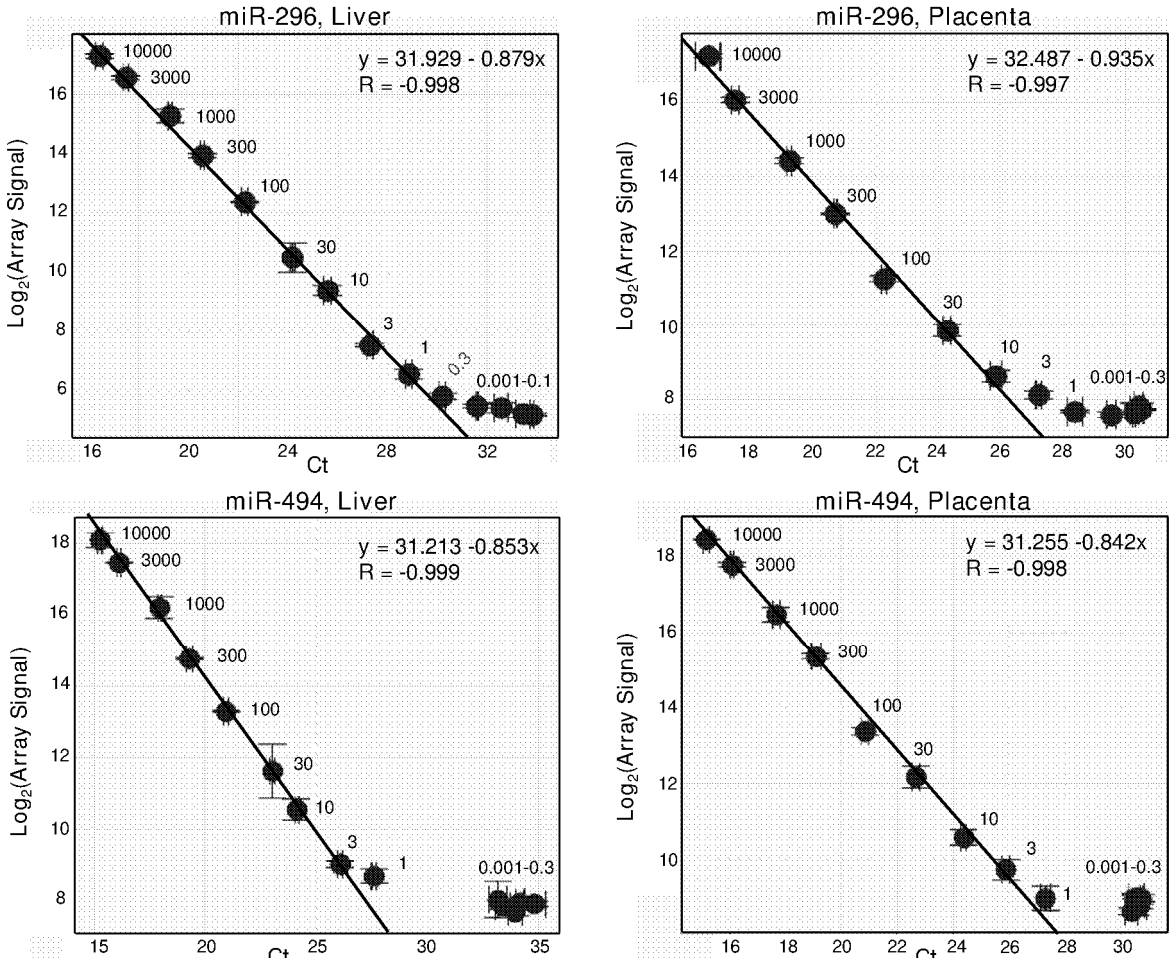


Figure 5. Comparison of qPCR and microarray measurements for miR-296 and miR-494 titrations. Scatter plots are shown for titration of synthetic miR-296 into liver (top left panel) and placenta (top right panel) total RNAs, and miR-494 into liver (lower left) and placenta (lower right) total RNAs. Ct values from qPCR are plotted on the x-axis, while $\log_2$ of the total gene signal from microarray measurements are plotted on the y-axis. Numbers in red show the number of attomoles of spike-in miRNA per 100 ng total RNA. Equations and R values are for the orthogonal line fit of the linear regions of each titration. Error bars indicate standard deviation (SD) of Ct values for qPCR results and (SD/Mean)*$\log_2$e for the array results.

Table 1. List and characteristics of sample preps from HeLa and ZR-75-1 cell pellets

Cell	Prep	No.	of RNA	Yield 260:280	Mean 260:230	Mean RIN	Mean

Line	Type	Preps	Mean (SD)	(SD)	(SD)	(SD)
HeLa	TRIzol	10	45.59 (7.17)	1.89 (0.0406)	1.28 (0.208)	9.9 (0.14)
HeLa	*mir*Vana	8	43.16 (12.01)	1.96 (0.0364)	1.36 (0.323)	9.9 (0.11)
HeLa	miR-Neasy	11	34.07 (5.40)	2.08 (0.00874)	1.97 (0.389)	9.9 (0.11)
ZR-75-1	TRIzol	4	23.52 (1.39)	1.84 (0.0432)	0.815 (0.0759)	9.8 (0.075)
ZR-75-1	*mir*Vana	4	16.92 (5.84)	2.01 (0.0356)	1.28 (0.361)	9.1 (0.17)
ZR-75-1	miR-Neasy	4	18.37 (1.93)	2.05 (0.0311)	1.87 (0.337)	9.8 (0.050)

The number of individual preps performed using the indicated total RNA prep method and cell lines are shown, as are the mean and standard deviations (in parentheses) of the total RNA yields (in micrograms), 260:280 ratios, 260:230 ratios, and RIN numbers for all the preps done with the same method in each cell type. Preps started with either 5 X 10^6 cells (HeLa) or 1 X 10^7 cells (ZR-75-1).

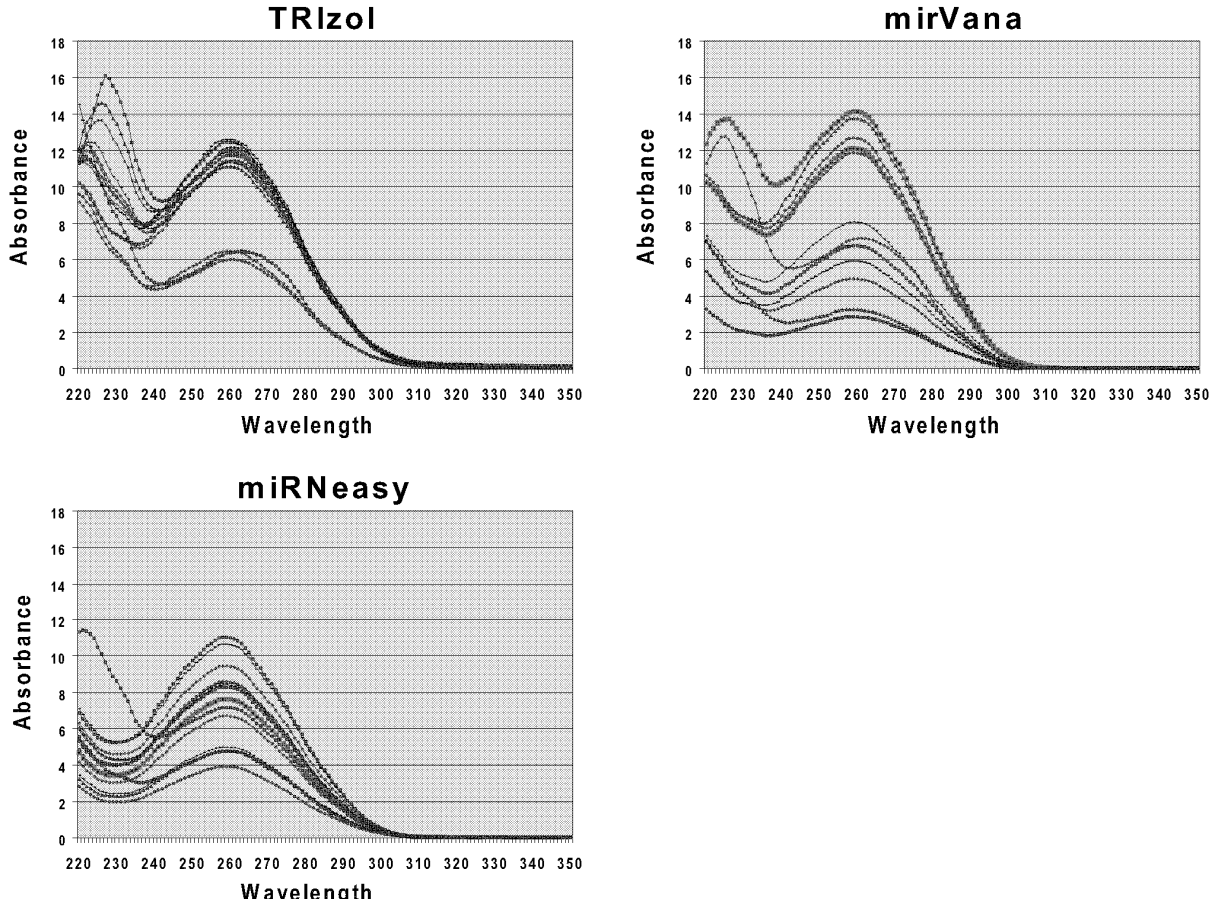


Figure 6. Absorption Spectra of HeLa and ZR-75-1 Sample Preps. The absorption spectra for the total RNA sample preps (listed in Table 1) are shown. Blue traces are HeLa cell preps and red traces are ZR-75-1 cell preps. Spectra are from 220 to 350 nm.

presence of contaminant(s). While this is most consistently seen in the TRIzol preps, it is also sometimes seen in the *mir*Vana preps and in one of the miRNeasy preps. In some

of the samples where no distinct peak is observed between 220–230 nm, the spectra show significant absorbance in the wavelengths immediately below 220 nm, with a shoulder tailing from 220 nm to 240 nm. At 240 nm the absorption increases again to peak at around 260 nm, which is the wavelength of maximum absorption for nucleic acids of mixed oligonucleotide composition. For many of these spectra, this absorption pattern suggests that the absorbance of the contaminant(s) whose peak is below 240 nm may overlap with the nucleic acid absorbance peak at 260 nm, which would result in the overestimation of nucleic acid quantity as determined by the absorption at 260 nm. Also, a couple of the absorbance spectra show a slight shoulder in the 260–270 nm range, indicating a contaminant which could also affect RNA quantitation. Thus, careful examination of sample spectra can be important for identifying samples where measured miRNA levels might be compromised by absorbance-based RNA quantitation artifacts.

Variability of hybridization results is highest between different prep methods

For miRNA microarray profiling analysis we took three of the replicate total RNA preps of each different prep method in each cell type and hybridized them to Agilent miRNA microarrays. A total of 42 hybridizations were done, with all but two samples hybridized at least twice (Additional File 16).

A good measure of the reproducibility of replicate measurements is the Root Mean Square (RMS) deviation of the natural logs of all signals that are well above background levels. The RMS deviation is approximately equal to the coefficient of variation of signals, and is an estimate of the proportional error of the measurement. For example, if the RMS deviation is 0.15 (15%), then a measured fold change between samples of 1.15 is a difference of one standard deviation.

We compared the RMS deviations between pairs of hybridizations performed using the same total RNA prep (hybridization replicates) with those performed using different total RNA preps done with the same method (prep replicates), and also with those using different total RNA prep methods. We also compared hybridizations performed on the same or different days, in order to take into account any day-to-day variability in the results. Figure 7a shows box plots of the RMS deviations between all possible replicate hybridization pairs, categorized by same or different prep method, same or different prep replicate, same or different hybridization replicate, and same or different hybridization day. The box plots for the six different categories are shown in decreasing order of variability. Hybridizations using preps from different methods show the most variability, with same day hybs of the different prep methods showing slightly less variability than different day hybs. Replicate preparations using the same method are the next lowest in variability, again with same day hybs being less variable than different day hybs. Finally, hybs done with aliquots of the same preparation have the lowest variability, again with same day hybs being less variable than different day hybs. The three sources of variability can thus be put in order of their magnitude: different prep methods > different preps using the same method > different hybridization day.

We also plotted the RMS deviations for hybridizations involving prep replicate pairs from each of the three different prep methods (regardless of the hybridization day), in order to examine whether the different prep methods showed different amounts of variation between prep replicates (Figure 7b). The TRIzol prep replicates showed less variability than the other two prep methods. A Student's ttest between pair-wise comparisons of the three prep methods confirmed that this difference is statistically significant (data not shown). There was no significant difference in variability between the mirVana and miRNeasy prep replicates.

A small subset of miRNAs differ between prep methods

To examine whether there are systematic differences among the miRNA profiles

observed for RNA isolated by different methods, we first looked at the overall signal levels of the hybridizations. The grand means of the mean total gene signal for all hybridizations of the same prep type for each cell line are shown in Table 2. For both cell types, the miRNeasy preps gave about 25% higher overall signals than the other two prep methods. The overall signal differences between the TRIzol and *mir*Vana preps of the same cell type were minimal. It should be noted that the miRNeasy preps had the highest 260:230 ratios (Table 1), and their spectra generally showed lower absorbance in the 220–230 nm range compared to the other two prep types. It is possible that some of the material which is absorbing at 220–230 nm in RNA extracted using the other two methods is contributing to the 260 nm peak, and it is also possible that DNA contaminants are present. The presence of either or both types of contaminant can lead to an overestimation of the amount of RNA present in these preps.

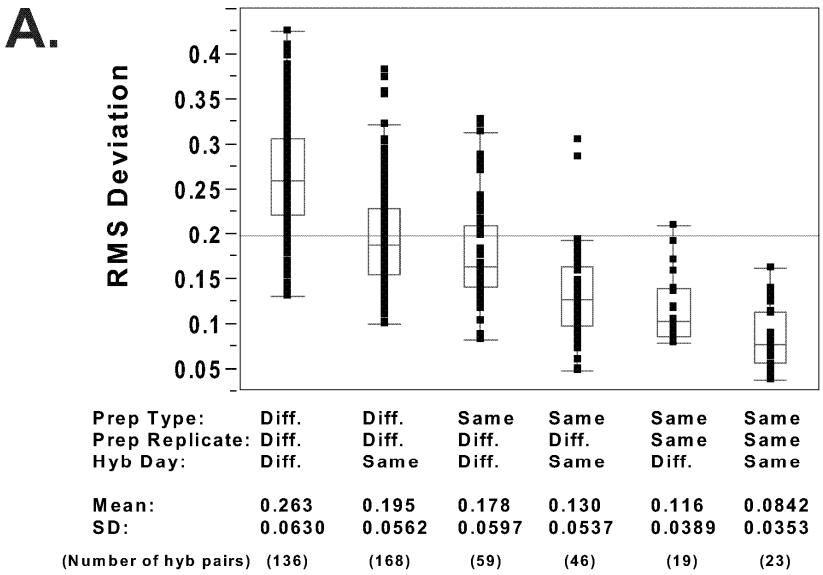
We next examined whether specific miRNAs systematically differ among the different prep methods (Figures 8 and 9). We calculated an average expression profile for each prep method for the two cell lines, by first averaging the total gene signals for each miRNA from all hybridizations of the same RNA prep, and then averaging together these individual prep averages for all preps of the same prep and cell type. Since there were differences seen in the overall signal levels between the different preps (Table 2), we normalized each pair-wise comparison to the 75^{th} percentile of one of the pairs. The expression profile of most miRNAs in HeLa cells does not depend on the RNA prep method (Figure 8). However, there is a small subset of miRNAs that consistently report different relative expression levels depending on the prep method. The miRNAs that are labeled in the figure show expression levels that differ by at least 2-fold in different prep methods. Additional File 17 lists the miRNAs which are 1.5x and 2x higher in one prep type compared to another. Three miRNAs are found at consistently lower levels in TRIzol preps than in the other two preps: miR-29b, miR-33, and miR219. *mir*Vana preps show consistently higher levels of four miRNAs when compared to the other two HeLa preps: miR-149, miR-328, miR-574, and miR-766. Figure 9 (and Additional File 17) shows the results from the ZR-75-1 breast cell line. While fewer miRNAs show different profiles among the three different prep methods in this cell line compared to HeLa, four out of the five that are observed to be discrepant in ZR-75-1 cells are the same as those seen in HeLa cells (miR-29b, miR-33, miR-219, and miR-328).

The finding that a small number of miRNAs report different microarray signals when prepared by different methods raises the question of whether these differences reflect real differences in the concentrations of these miRNAs in the different sample preps. To examine this, we assayed individual samples prepared with the three methods by qPCR, using primers for three of the miRNAs showing differences between the prep methods. We then compared these results to those obtained from microarray analysis of the same preps (Figure 10). The good agreement of the qPCR results with the array results for these miRNAs strongly suggests that the differences in the miRNA levels observed between the sample prep methods reflect true differences in the miRNA content of the extracted RNA, and are not artifacts of the measurement assay. At present, we have no explanation for why these particular miRNAs are found at different levels when using different extraction techniques.

Conclusion

In this study we compared the expression levels of 61 miRNAs in nine human tissues as measured by both Agilent microarrays and TaqMan qRT-PCR. We found that 53/60 expressed miRNAs had correlations (R) > 0.9 between the two methods. For the two miRNAs that differed most between the two methods, spike-in studies found the differences are not due to differential sensitivity of the two methods, but are more likely due

to interference from other RNAs in the com- plex mixture.



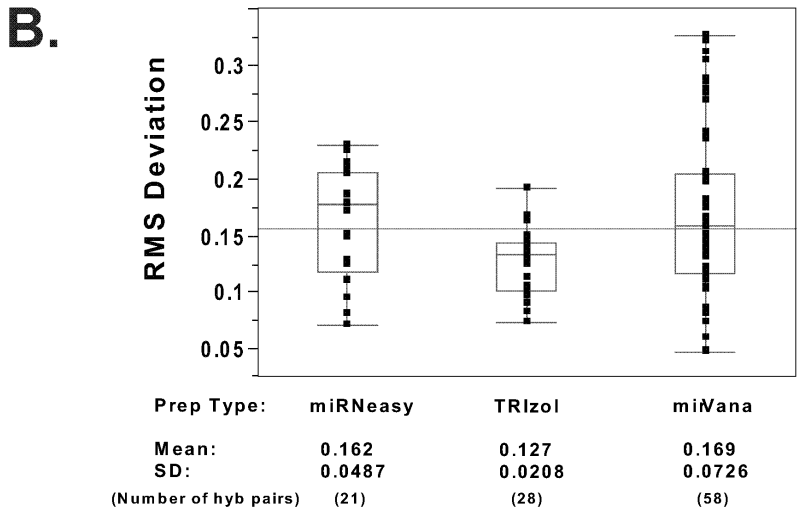


Figure 7. Box plots of RMS deviations for microarray hybridization pairs. (a) RMS deviations of all possible hybridization pairs of the same cell type. Box plots show the distribution of RMS deviations calculated from all possible pairs of hybridizations done with the same cell type, and sorted by prep type, prep replicate, and hybridization day. Plots show the combined results from HeLa and ZR-75-1 cells. Each replicate pair RMS deviation is indicated by a black dot. The ends of the box represent the 25^{th} and 75^{th} quartiles, while the line through the center of the box represents the median. The whiskers from each box extend to the outermost data point included in the range from the upper quartile plus 1.5*(interquartile range) to the lower quartile minus 1.5*(interquartile range). The line across the entire plot indicates the mean of all the values. Values of the mean and standard deviations for each set of values are shown at the bottom of the plot. (b) RMS deviations of all prep replicate hybridization pairs, sorted by prep type. Box plots of the RMS deviations for all the pair-wise comparisons of hybridizations using replicates of the same prep type, and sorted by prep type. Plots show the combined results from HeLa and ZR-75-1 cells. Box plot details are as in Figure 7a.

We also examined microarray-based miRNA profiles using three different total RNA sample prep methods. We found that while almost all miRNA levels correspond

between the three different prep methods, a small subset of 2–10 miRNAs consistently differ by greater than 2-fold between different techniques. These differences were corroborated using qPCR, and are most likely due to true differences in the miRNA content of the extracted RNA. Thus, while all three methods are suitable for use in profiling miRNAs from total RNA, it may be prudent to pick one method and use it for the entire course of any particular study, in order to avoid these small profile differences due to the RNA preparation method.

Table 2. Grand means of the mean total gene signal for all hybridizations of each prep type

Cell Line	Prep Type	Mean TGS (SD)
HeLa	TRIzol	235.0 (19.1)
HeLa	*mir*Vana	211.2 (25.8)
HeLa	miRNeasy	290.3 (19.2)
ZR-75-1	TRIzol	407.9 (33.0)
ZR-75-1	*mir*Vana	437.1 (84.5)
ZR-75-1	miRNeasy	546.7 (41.9)

The mean of the total gene signal for all the miRNAs on the microarray were calculated for each individual hybridization, and the mean and standard deviation of these for each prep type in each cell line are shown.

Methods

Total RNA and cell samples

Total RNA samples from normal human tissues were from Ambion (Austin, TX). Frozen HeLa cell pellets were from Cell Trends (Middletown, MD), and frozen ZR-75-1 cell pellets were from BioProcessing Inc. (Portland, ME).

miRNA microarray analysis

miRNA microarrays were manufactured by Agilent Technologies (Santa Clara, CA)., and contain 20–40 features targeting each of 470 human miRNAs (Agilent design IDs 015508 (sample prep studies) and 016436 (nine tissue comparison studies)) [37]. Sequences of the 470 miRNAs were obtained from the Sanger miRBase, release 9.1 [1012]. Labeling and hybridization of total RNA samples were performed according to the manufacturer's protocol. 100 ng total RNA was used as input into the labeling reaction, and the entire reaction was hybridized to the array for 20 hours at 55°C. For the microarray versus qRT-PCR comparisons, the labeling and hybridizations of the nine human tissues were done 4–5 times, and the mean and standard deviation for each miRNA were calculated.

Microarray results were extracted using Agilent Feature Extraction software (v9.5.3.1) and analyzed using GeneSpring GX 7.3.1 software (Agilent Technologies) and Spotfire DecisionSite 8.1 software (TIBCO Software, Palo Alto, CA). Box plots were calculated using JMP 5.1 software (SAS, Cary, NC). Original microarray data is deposited in the Gene Expression Omnibus [38] (Series GSE11879).

All scatter plots of miRNA microarray data use the total gene signal, which is proportional to the total number of targets bound by the probes targeting each miRNA [31,32]. For comparison of two hybridizations, the natural logs of the total gene signals for all genes expressing above 10x the background noise in both samples were regressed against each other, and the standard deviation of the residuals from the regression line were reported as the RMS deviation. For most pairs of samples prepared by the same method, residuals were normally distributed, so that the RMS deviation describes true random variation in the assay. In pairs of samples prepared by different methods, residuals of most of the miRNAs were also normally distributed, with systematic exceptions of some miRNAs as discussed in the text. No normalization was performed for either microarray or qPCR data, except for an overall intensity normalization applied to the average signals from different prep methods, as described in the text (Figures 8 and 9). For this comparison, the 75th percentile of the total gene signal for all the miRNAs on the array was calculated by sorting the total gene signals for 470 miRNAs on the array in ascending order, and the signals from the three methods were normalized to the signal from the 353rd miRNA.

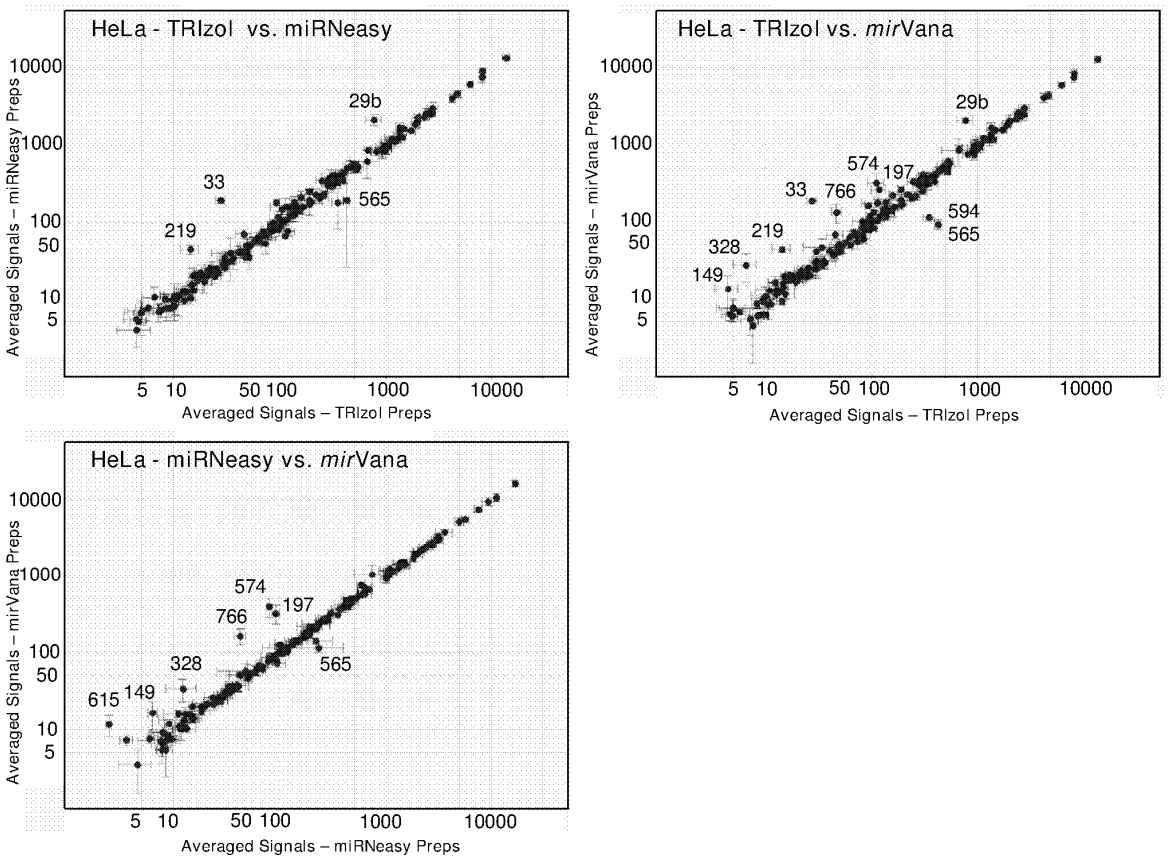


Figure 8. Pair-wise comparisons of averaged profiles of the three different prep types: HeLa cells. Total gene signals for each miRNA for all hybridizations of the same RNA prep (hybridization replicates) were averaged, and then these averaged individual prep profiles for all the preps of the same prep type were averaged together to get a mean profile for each of the three prep types. Scatter plots show these averaged profiles from one prep type plotted against another for HeLa cells. Error bars indicate one standard deviation. Numbers indicate the identity of all miRNAs whose signal strengths are at least two-fold higher in one prep type than another, after normalization.

miRNA qRT-PCR analysis

miRNA qRT-PCR analysis was performed using Taqman miRNA assays (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. 5 ng total RNA was input into each reverse transcription reaction (RT) for each miRNA. Four replicates were done for each miRNA, consisting of two replicate PCR reactions from each of the two replicate RT reactions, and the results were averaged. PCR reactions were run on a 7500 Real Time PCR machine (Applied Biosystems) and analyzed using 7500 System SDS software (v1.4).

miRNA spike-ins

Synthetic miRNAs were manufactured by TriLink BioTechnologies (San Diego, CA) and spiked into human liver and placenta total RNA (Ambion). 100 ng of these RNA mixes were then used for labeling and hybridization onto the microarrays, while 5 ng were used as input into the reverse tran-

scriptase reaction for qPCR. Two replicate microarray hybridizations and four replicate qPCR reactions were done for each dilution in each tissue.

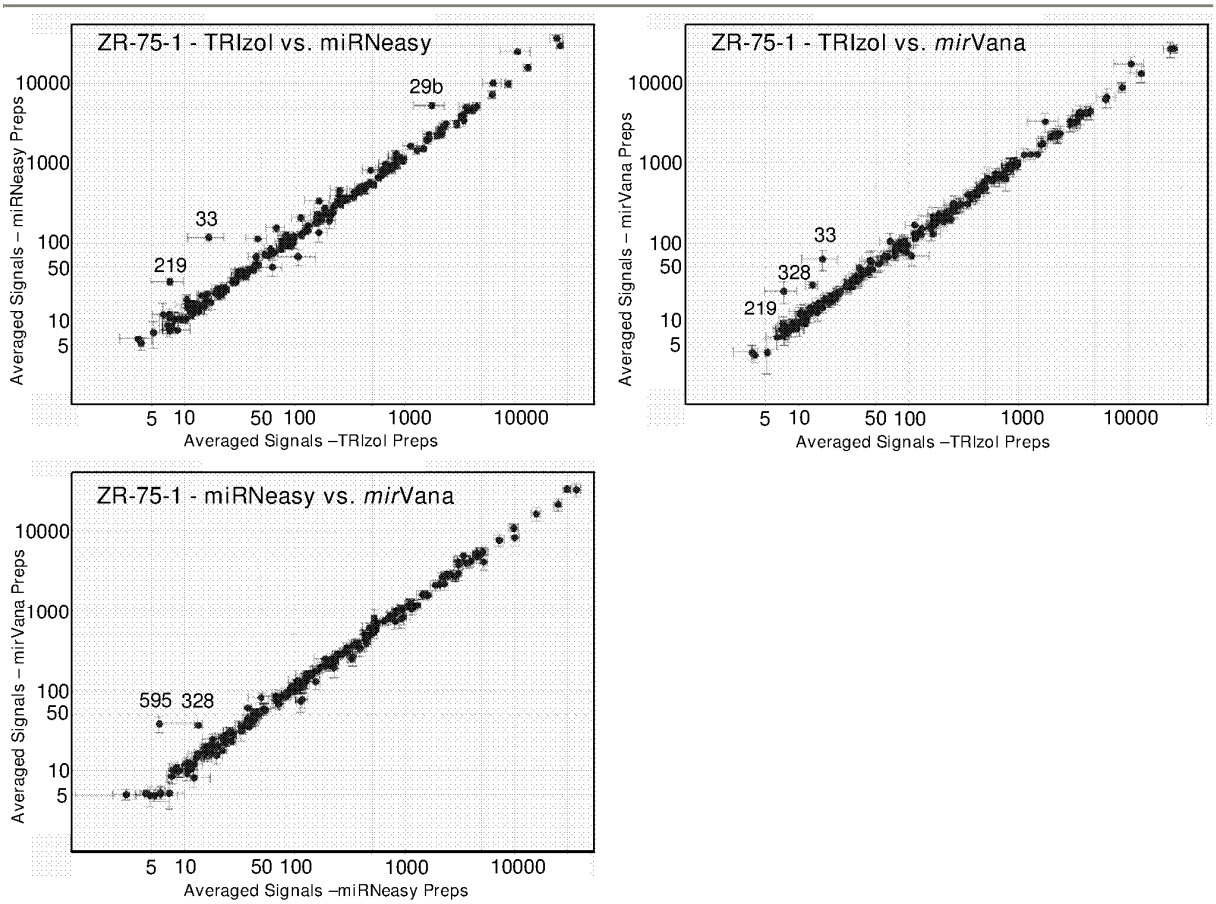


Figure 9. Pair-wise comparisons of averaged profiles of the three different prep types: ZR-75-1 preps. Scatter plots as in Figure 8, but for ZR-75-1 cell preps.

Total RNA sample preps

Frozen cell pellets were resuspended in phosphate buffered saline and divided into equal aliquots of 5 X 10^6 (HeLa) or 1 X 10^7 (breast) cells and refrozen. Individual aliquots were subsequently thawed just before use.

TRIzol preps were performed according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) using an isopropanol precipitation. Briefly, 1 ml of TRIzol reagent was added to the cell pellet and cells were lysed by repetitive pipetting, and then incubated at room temperature for 5 minutes. 200 µl of chloroform were added, followed by vigorous shaking and incubation for 2–3 minutes at room temperature. Samples were centrifuged 15 minutes at 12000 X g at 4°C. The aqueous layer was transferred to a new tube, and the RNA was precipitated by adding 0.5 ml isopropanol, incubating 10 minutes at room temperature, and spinning for 10 minutes (12000 X g at 4°C). Pellets were washed with 80% ethanol and resuspended in nuclease-free dH_2O (Ambion).

miRNeasy total RNA preps (QIAGEN, Valencia, CA) were performed according to the manufacturer's protocol. The *mir*Vana miRNA Isolation kit (Applied Biosystems) was used according to the manufacturer's protocol for total RNA isolation.

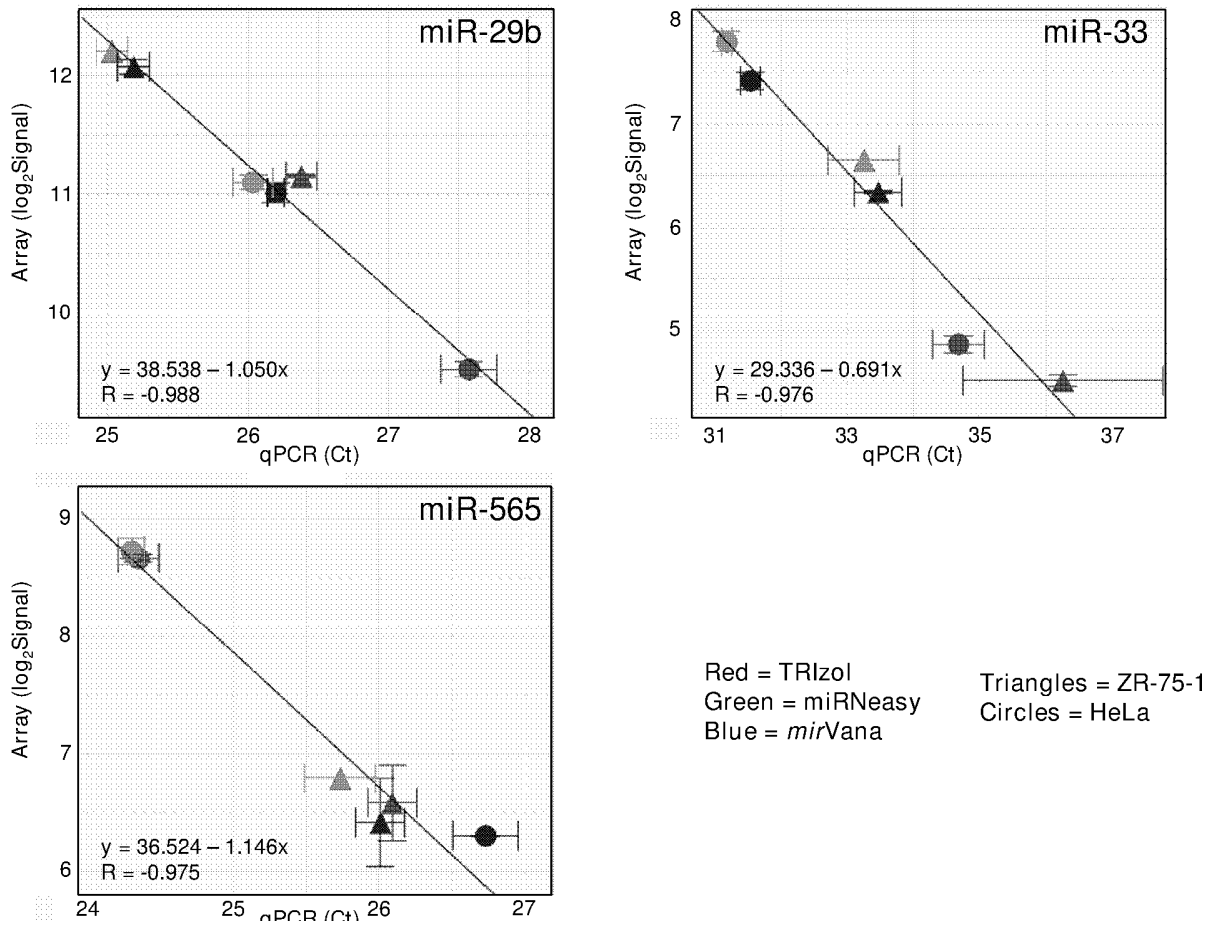


Figure 10. Comparison of qPCR and microarray miRNA results for three miRNAs differentially measured between different prep types. Individual TRIzol, miRNeasy, and *mir*Vana preps were assayed with qPCR for three miRNAs found in microarray studies to be at higher levels in one prep type than another. Scatter plots show qPCR results (cycle threshold (Ct) values) on the x axes and microarray results (log_2 of the total gene signal) on the y axes. Each data point represents one individual prep from one cell type. Circles indicate HeLa preps and triangles represent ZR-75-1 preps. TRIzol preps are in red, miRNeasy preps are in green, and *mir*Vana preps are in blue. The equations and R values on each plot are for the line of best fit. Error bars indicate standard deviation (SD) of Ct values for qPCR results and (SD/Mean)*log_2e for the array results. Note that the axes are not on the same scale in the three different plots.

All total RNA preps were analyzed using the 2100 Bioanalyzer (Agilent Technologies), RNA 6000 Nano LabChip kits, and 2100 expert software (version B.02.05.SI360). Absorption spectra were measured on an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Additional material

Additional File 1

qRT-PCR Ct data and microarray signal data for 61 miRNAs. *Mean Ct values data for the four qPCR replicates are listed, as are mean microarray data (log_2 of the total gene signal) for the microarray replicates.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S1.xls]

Additional File 2

Comparison of qPCR and microarray miRNA profiling for individual miRNAs. *Scatter plots for 51 miRNAs not shown in Figure 1.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S2.pdf]

Additional File 3

Comparison of qPCR and microarray miRNA profiling for individual miRNAs. *Scatter plots for 51 miRNAs not shown in Figure 1.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S3.pdf]

Additional File 4

Comparison of qPCR and microarray miRNA profiling for individual miRNAs. *Scatter plots for 51 miRNAs not shown in Figure 1.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S4.pdf]

Additional File 5

Comparison of qPCR and microarray miRNA profiling for individual miRNAs. *Scatter plots for 51 miRNAs not shown in Figure 1.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S5.pdf]

Additional File 6

Comparison of qPCR and microarray miRNA profiling for individual miRNAs. *Scatter plots for 51 miRNAs not shown in Figure 1.*

lementary/14726750-8-69-S6.pdf]

Additional File 7

Comparison of qPCR and microarray miRNA profiling for individual miRNAs. *Scatter plots for 51 miRNAs not shown in Figure 1.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S7.pdf]

Additional File 8

Comparison of qPCR and microarray miRNA profiling for 60 miRNAs in tissue pairs. *Scatter plots for 32 tissue pairs not shown in Figure 3.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S8.pdf]

Additional File 9

Comparison of qPCR and microarray miRNA profiling for 60 miRNAs in tissue pairs. *Scatter plots for 32 tissue pairs not shown in Figure 3.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S9.pdf]

Additional File 10

Comparison of qPCR and microarray miRNA profiling for 60 miRNAs in tissue pairs. *Scatter plots for 32 tissue pairs not shown in Figure 3.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S10.pdf]

Additional File 11

Comparison of qPCR and microarray miRNA profiling for 60 miRNAs in tissue pairs. *Scatter plots for 32 tissue pairs not shown in Figure 3.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S11.pdf]

Additional File 12

Comparison of qPCR and microarray miRNA profiling for 60 miRNAs in tissue pairs. *Scatter plots for 32 tissue pairs not shown in Figure 3.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S12.pdf]

Additional File 13

Comparison of qPCR and microarray miRNA profiling for 60 miRNAs in tissue pairs. *Scatter plots for 32 tissue pairs not shown in Figure 3.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S13.pdf]

Additional File 14

Comparison of qPCR and microarray miRNA profiling for 60 miRNAs in tissue pairs. *Scatter plots for 32 tissue pairs not shown in Figure 3.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S14.pdf]

Additional File 15

Comparison of qPCR and microarray miRNA profiling for 60 miRNAs in tissue pairs. *Scatter plots for 32 tissue pairs not shown in Figure 3.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S15.pdf]

Additional File 16

List of microarray hybridizations done with HeLa and ZR-75-1 preps. *Three or four preps done with each method (labeled A-D) were hybridized for the indicated number of times.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S16.xls]

Additional File 17

List of miRNAs measured at higher levels in one prep type over another. *miRNAs found at levels either > 2x or between 1.5x and 2x in one prep type over another, in HeLa and ZR-75-1 total RNA preps. Ratios are the ratio of normalized total gene signals on microarrays in prep 1 versus prep 2.*

[http://www.biomedcentral.com/content/supplementary/14726750-8-69-S17.xls]

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AMBIVALENCE RELATED TO POTENTIAL LIFESTYLE CHANGES FOLLOWING PREVENTIVE CARDIOVASCULAR CONSULTATIONS IN GENERAL PRACTICE: A QUALITATIVE STUDY

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Background: Motivational interviewing approaches are currently recommended in primary prevention and treatment of cardiovascular disease (CVD) in general practice in Denmark, based on an empirical and multidisciplinary body of scientific knowledge about the importance of motivation for successful lifestyle change among patients at risk of lifestyle related diseases. This study aimed to explore and describe motivational aspects related to potential lifestyle changes among patients at increased risk of CVD following preventive consultations in general practice.
Methods: Individual interviews with 12 patients at increased risk of CVD within 2 weeks after the consultation. Grounded theory was used in the analysis.
Results: Ambivalence related to potential lifestyle changes was the core motivational aspect in the interviews, even though the patients rarely verbalised this experience during the consultations. The patients experienced ambivalence in the form of conflicting feelings about lifestyle change. Analysis showed that these feelings interacted with their reflections in a concurrent process. Analysis generated a typology of five different ambivalence sub-types: perception, demand, information, priority and treatment ambivalence.
Conclusion: Ambivalence was a common experience in relation to motivation among patients at increased risk of CVD. Five different ambivalence sub-types were found, which clinicians may use to explore and resolve ambivalence in trying to aid patients to adopt lifestyle changes. Future research is needed to explore whether motivational interviewing and other cognitive approaches can be enhanced by exploring ambivalence in more depth, to ensure that lifestyle changes are made and sustained. Further studies with a wider range of patient characteristics are required to investigate the generalisability of the results.

Background

Psychological [1-7], sociological [8-11], medical [12-23], educational [24,25] and anthropological [26,27] traditions have all contributed extensively to our knowledge about lifestyle change processes. They have identified key psychological constructs such as different kinds of resistance mechanisms related to motivation and lifestyle change [28-30]. Since 1999, the Danish College of General Practitioners has dedicated 'preventive consultations' comprising motivational interviewing (MI) [7,31] as an approach in the prevention of lifestyle related diseases such as CVD. The preventive consultation is a scheduled consultation focusing on individual prevention and risk reduction strategies, where the person is aware of the agenda such as diet, physical activity, smoking, alcohol or other issues in advance and therefore able to prepare himself for the consultation. An agreement is sought about treatment goals to meet public health priorities towards decreasing the risk of: diabetes, CVD, cancer, osteoporosis, chronic obstructive lung disease, asthma, chronic muscle diseases and mental health conditions [31]. MI is a client-centred and supportive counselling method aimed at enhancing readiness for change by eliciting the client's own motivations for

change [7]. The evidence base for MI is strong in the areas of addictive and health behaviour and in research settings MI has out-performed "traditional advice giving" in the treatment of a broad range of behavioural problems and diseases [12]. Furthermore, meta-analysis has shown a significant and clinically relevant effect for MI on combined risk factor profiles including body mass index, total cholesterol, blood pressure, alcohol consumption and a significant, in approximately three out of every four studies. [12] Forty-six out of 72 trials also showed benefits on individual lifestyle risk factors with most success for obesity and alcohol consumption, and less effect on smoking [12]. Improvements were more likely, when more than one encounter had taken place, but further studies were recommended to examine the implementation and effectiveness of MI in daily clinical work [12]. However, little is known about the motivational content in preventive cardiovascular consultations in general practice or their general effectiveness in achieving lifestyle change and risk reduction.

The present study is inspired by current recommendations to use MI in primary preventive strategies and the empirical and multidisciplinary body of scientific knowledge on the importance of motivational aspects regarding potential lifestyle changes. The aim of the study was to explore and describe motivational aspects related to potential lifestyle changes in preventive consultations in general practice among patients at increased risk of CVD. This study presents ambivalence and a typology of subtypes as the core motivational aspect among patients at increased risk of CVD following preventive consultations with their GPs.

These GPs had not been specifically educated or trained in MI but introduced to MI through guidelines by the Danish College of General Practitioners on how to use MI in clinical practice.

Methods

This qualitative study draws its data from 12 one-to-one interviews conducted within 2 weeks after preventive consultations, which were videotaped. In all 30 GPs were included from the Health Insurance Register in Vejle and Aarhus Counties and sampled purposefully in relation to age, gender, communicative education and preventive consultation activity. This helped us to ensure that the sample reflected the range of GPs involved in the daily care of patients at increased risk of CVD on the basis of their preventive service experience and the public guidelines. Seven female and five male GPs mostly from group practices participated. The GPs had worked as practitioners for an average of 12.8 years and their average age was 47.7 years. Three of 12 GPs had prior education and training in MI, another three had psychological training from Balint groups and one from a cognitive therapy course.

The 12 participating GPs recruited 12 patients purposefully in accordance with the risk criteria: 20% or higher risk of contracting CVD within the next 10 years, assessed by the risk score system Precard [32] and variability in the person criteria: gender, age and education selected to address the purpose of the study and to gather information rich data. PRECARD is the model for the Heart Score software, developed by the European Society of Cardiology, which aims to provide detailed risk assessment tools in the area of cardiovascular disease to European countries in general [32]. Purposeful sampling implies that participant and other data sources are chosen, because of their importance to the purpose of the study [33].

Table 1 shows the patients' characteristics and summarises their actual risk factors that were being addressed in the consultations. Two women and 10 men participated; their average age was 57.8 years and they came from different social classes and had attained varying educational levels.

A pilot interview study (n = 3) was conducted during the development of the interview guide focused on motivation [Table 2], which was continually modified as new themes emerged from the data. The first author conducted the one-hour interviews

within 2 weeks after the consultation, because we aimed to describe motivational aspects of lifestyle change immediately after the consultation and to reduce recall bias related to memory of the consultation at later stages. Before each interview the research interviewer saw the videotaped consultation belonging to the specific person (mean duration 18 minutes) in order to inform and 'qualify' the interview guide, i.e. to ensure that the subsequent interview with the patient addressed issues relevant to his/her specific consultation. For instance when interviewing about influential motivational factors, the videotaped consultations were used to qualify and focus the interview guide on the specific risk factors considered in the consultation.

Table 1 Characteristics of the patients

Informants	Gender	Age	Employment	Risk factors	Co-morbidity
1	♂	74	Factory worker and pensioner	Hypertension, Hypercholesterolemia, Ex-smoker and Overweight	None
2	♂	69	Taxi owner and pensioner	Hypertension, Smoker, Hypercholesterolemia and IGT*	None
3	♀	57	Factory worker, early retired	Hypertension, Hypercholesterolemia Smoker, Overweight and IGT*	Fibromyalgia
4	♂	43	Drilling platform worker	Smoker, Hypercholesterolemia and Overweight	Knee problems
5	♂	73	Engineer and pensioner	Hypertension, Hypercholesterolemia and Ex-smoker	None
6	♂	51	Interpreter	Hypertension, Hypercholesterolemia and Smoker	None
7	♀	42	Architect and manager of the firm	Hypercholesterolemia, Overweight and Hypertension	None
8	♂	54	Manager in the provision industry	Hypercholesterolemia, Hypertension, Smoker, Overweight and IGT*	None
9	♂	49	Gardener	Hypercholesterolemia, Hypertension, Overweight and Ex-smoker	None
10	♂	69	Grocer and pensioner	Hypercholesterolemia, Hypertension and Smoker	None
11	♂	48	Manager and municipal politician	Hypercholesterolemia, Hypertension, Smoker and overweight	None
12	♂	65	Dock worker and pensioner	Hypercholesterolemia, Hypertension and Overweight	None

*Insuline glucose intolerance

Consequently the videotaped consultation was not used as a primary data source. The patients did not see the video before the interview. During the interviews, the patients were first asked to recall whatever they remembered from the consultation and how they felt about it. They were then encouraged to describe their experiences and to explore, in particular, their readiness to change lifestyle. They were prompted to address five groups of questions addressing motivational aspects shown in table 2. The interviews were transcribed verbatim by a trained secretary and the first author, read and coded independently and discussed after coding of each interview with the last author of the article, an experienced qualitative researcher.

Analysis

Coding of themes followed the objectivistic rules of Grounded Theory [33,34] and was carried out through four phases supported by the software program N-vivo 2.0. In the *open coding phase*, the 'motivational phenomena' present in the interview quotations were coded and divided into categories and sub-categories. In the *axial coding phase*, each category was analysed in order to identify its dimensions and characteristics. In the *selective coding phase*, the main categories were generated and named, given their empiric contents. Finally the ambivalence subtypes were generated and described under the overall concept of ambivalence, which was identified as the core motivational aspect (for further details see Table 3). Throughout these four phases the first author (DK) carried out the initial coding of the interviews. Subsequently, the last author (MBR) also read and identified all codes from the interviews then meeting to discuss, add or revised the coding and analytical core categories of each interview by consensus.

Table 2. Motivational questions in the interview guide

Questions to patients	
Introductive	How do you understand the word motivation? Describe what you remember from the consultation about motivation and how you felt about it?
Question 1	Did you feel ready to accept your GP's preventive consultation offer?
Question 2	How ready were you to change lifestyle before and after the consultation? a. Tell me about your readiness to change lifestyle before the consultation? b. Tell me about your readiness to change lifestyle after the consultation? c. If you were not ready to change lifestyle, then tell me why?
Question 3	Which aspects influence your motivation to change lifestyle in general and in the consultation? a. Which aspects or factors make you ready to change lifestyle? b. Which aspects or factors inhibit your readiness to change lifestyle?
Question 4	Describe how your GP tried to motivate you in the consultation and what you felt about it?
Question 5	Which persons or networks have the greatest influence on your readiness to change lifestyle?

Data were collected, prepared, analysed and interpreted in a concurrent repetitive process involving the empirical material (researcher and participants constructions of the consultation), inspiring theoretical aspects and the study objectives in line with the constructivist grounded theory approach [34] and the so-called analytical "round dance" [35]. By the term "analytical round dance" is meant that the qualitative process from the data collection phase to the final analytical interpretation of the empiric data process is a dynamic and fluid process where the empiric data interacts with the methodological strategies such as for instance the purposeful sampling and the constant comparative strategy and the relevant theories such as for instance motivational interviewing and other behavioural change theories at each step of the analysis. Given the grounded theory method, the focus of the interviews developed and became more theoretically specific as the sequence of interviews progressed; that is, data capture was driven by emerging categories and theory development [34].

During the conduct and analysis of the 12 interviews, the emerging categories were examined for theoretical saturation [33,34] i.e. to examine whether further comparisons, properties or relationships developed or new theoretical insights were revealed [34] (but not for saturation to achieve representativeness).

Results

Our analysis identified that ambivalence and different ambivalence subtypes: *perception, demand, information, priority and treatment* ambivalence were the core motivational aspects related to lifestyle change following preventive consultations. The following section will describe these subtypes as the main results of our study and offer quotations to illustrate common themes.

Perception ambivalence

Perception ambivalence captured ambivalent feelings and reflections related to patients' perception of being at risk of CVD, on the one hand, and their perception of being healthy or sick on the other. For instance a 42-year old female found it difficult to separate her perception of being at risk from being healthy or suffering from a CVD:

It is a difficult situation when you are on your way to an unhealthy lifestyle. When is it unhealthy and when is it not? When do you have to stop and prevent heart disease?

How do you separate risk from being healthy and/or suffering from cardiovascular disease? How do I convince myself about the fact that I should act preventively, when I feel well? These conflicting feelings and

thoughts fill my head after the consultation. (id 7)

Our analysis of patients' perception ambivalence furthermore showed that the conflicting (ambivalent) feelings seemed to interact with the patients' reflections in a concurrent process. These reflections were person-specific and related to aspects such as knowledge, considerations and actions related to lifestyle change. A 69-year-old male informant said:

Table 3. Analytical coding phases in the conceptualisation of ambivalence and the different subtypes

Grounded Theory Coding phases	Descriptions	Categories
The open coding phase	Identified categories and their antagonistic relations	To be at high risk of cardiovascular disease, having cardiovascular disease, to be healthy, to be unhealthy, to know about illness, to know enough, to change life style, to live unchanged, to take medicine, to live without medicine, to add risk, not to add risk, preventive demands from the health care system, the GP or the family, preventive demands from the risk patient themselves, to contain risk, to act preventively, to know about risk, a lifestyle with stress and many demands, a lifestyle without stress and fewer demands, priority of free time and/or health and/or physical activity and/or family and/or resources
The axial coding phase	The common dimension of the categories and their characteristics	Conflicting feelings and reflections regarding: 1. To have cardiovascular disease versus to be at high risk of cardiovascular disease. 2. To be healthy versus to be unhealthy. 3. To know about risk/disease versus not to know. 4. To know about risk/disease versus to know enough. 5. To change lifestyle versus to live on without changes. 6. To take medicine versus to live without medicine. 7. To change lifestyle versus to take medicine. 8. To add risk to life versus to stay status quo. 9. Preventive demands from the health care system versus preventive demands from the risk patient. 10. Preventive demands from the general practitioner versus the demands from the risk patient. 11. Preventive demands from the network such as family versus the risk patient's own demands. 12. To know about risk versus to contain risk. 13. To contain risk versus to decide to do something about risk. 14. To know about risk versus to act preventively. 15. Priority of spare time or family or work or physical activity versus priority of the risk patients' own resources and health in every day life. 16. To live a stressed life with many demands versus to live an unstressed life with fewer demands
The selective coding phase	The antagonistic categories with their two dimensions were collected into main categories and named on behalf of their empirical characteristics leading to the main concept of ambivalence, its different sub-types and the concurrent reflective process	Main category 1: Perception ambivalence (sub-categories 1+2) Main category 2: Demand ambivalence (sub-categories 9–11 and 16). Main category 3: Information ambivalence (sub-categories 3+4, 12+14) Main category 4: Priority ambivalence (sub-category 15). Main category 5: Treatment ambivalence (sub-categories 5–8, 13)
The theory or concept generating phase	Definition and types of ambivalence	Ambivalence was defined by conflicting feelings that were found to interact with patients' reflections on lifestyle changing in an iterative and concurrent process. Our analysis brought forward five different ambivalence sub-types: perception, demand, information, priority and treatment ambivalence.

You feel stupid, when you consult the doctor and he confirms what you already know and think, and, even so, you are unable to act, because you are filled with conflicting feelings about lifestyle changes. The feelings disturb the thoughts. I know what it is all about – it is just so difficult to get my act together. Actually, to know about risk, health and lifestyle habits is very different from being able to consider ... and to consider risk is not always the same as being able to act preventively. (id 10)

Demand ambivalence

The *demand ambivalence* was due to conflicting demands from the health care system/the GPs/the family, on the one hand, and the patients' own demands on the other hand. This generated confusion about which demands patients should meet. A 65-year-old male informant said:

My views on risk of cardiovascular disease seem to be different from the demands from the health care system, my family or my doctor ..... If you cheat the doctor by just saying something you don't do, or pretend to do, you are cheating both the system and yourself. My doctor talked about the fact that we have to estimate my risk of dying from heart disease ..... For me risk of disease is something I should deal with when I grow old. I am still young and have no symptoms, so why should I comply with these demands? (id 12)

The patients experienced furthermore that the demand ambivalence often gave rise to strong personal feelings of stress, which was rooted in and arose from the many and conflicting demands from working life, family or the patients themselves, which affect their confidence and ability to implement lifestyle change. They explained how their feeling of stress eroded their focus on health, made them less aware of their body and more concerned. They felt caught in a vicious circle with limited control over their own lives; a situation where family, work and other external circumstances exercised more control than they did themselves. The informants described furthermore, how their demand ambivalence and feeling of stress could result in conflicting choices between a stressed life with much result-oriented activity versus an unstressed life with lower activity and subsequently fewer demands to achieve results. A 48-year-old male informant said:

Physically, I became increasingly inactive because of stress, work demands ..... Then concerns about my health increased and so did my weight. Then it was just more difficult for me to be physically active and I became more stressed and more passive about prevention – it became a vicious circle. I experienced being so stressed in periods that I did not listen to the signals from my body and lost my will. Suddenly, I was resistant to lifestyle changes. (id 11)

Information ambivalence

The *information ambivalence* captured uncertainty about how much information the patients actually preferred and from whom. The patients often used their GPs' information as a starting point immediately after the consultation, even though it made them ambivalent. A 69-year-old male informant said:

I don't really know how much information I need. Too much information could make me confused, too little information could make me unaware that I am at risk. I feel that my doctor's information is important, but it makes me unsure what to do. (id 2)

The information ambivalence also contained conflicting feelings and reflections on the sufficiency of information in the consultation, varying from one situation to another. As a 43-year-old male informant said:

Sometimes a little information about my health is sufficient if that's just what I need. Some other times I feel I need much more information. (id 4)

As well as the amount of information causing tensions, the type of information was also important. For instance, the patients found it difficult to interpret and respond to numbers offered in explanations of risk, con-

cepts and definitions. A 54-year-old male informant said:

I like a combination of approaches such as pictures, colours or figures combined with ordinary words and numbers. Then I feel informed. If my GP uses numbers to communicate complex medical risk concepts, then I don't feel informed in a way, because I cannot respond. Besides, how do I know if I am the one who goes free or the one who gets ill? ..... I just need ordinary words, numbers and visual information to feel informed. (id 8)

Priority ambivalence

Priority ambivalence was due to assigning priority in life in relation to working life, health, family and own life and resources.

Priority ambivalence derived for example from either a low commitment or inclination to prioritise health, or physical barriers such as back or knee pain, preventing the patients from changing their exercise habits. The work, family or social networks strained their resources and were given higher priority than their health. A low health priority was, furthermore, related to a low readiness to change lifestyle than a high health priority. A 49-year-old male informant said:

it is difficult to be physically active. Besides, I often experience that my family life and activities force me to reduce my own health preventive activities and spare time and ..... To change lifestyle is about setting your own targets. ..... Low priority of health goes against a healthy lifestyle. (id 9) and another 42 year-old female informant:

Every day you have to make priorities and with family and fulltime work it can be difficult to prioritize health in your daily living in practice. If you really want to change lifestyle you must prioritize it as an important agenda in your every day living. (id 7)

Treatment ambivalence

Treatment ambivalence typically consisted of ambivalent feelings and reflections about the need to change one's lifestyle and take medicines. Patients would prefer to adopt one or the other approach, but doctors often characterised it as a "both and" situation among high-risk patients. High-risk patients are typically recommended to change lifestyle and take treatment, but they do not always accept this – i.e. they are placed in an ambivalent position. The added risk of taking medicine refers especially to the risk of suffering from side effects versus changing lifestyle. This was frequently mentioned in the interviews. A 43-year-old male informant said:

It is just much easier to take medicine than to change lifestyle. If I could, I would prefer medical treatment, because lifestyle changes are so difficult. On the other hand, taking medicine also carries a risk of side effects – an added risk. (id 4)

After a preventive consultation, many patients identified issues that reflected ambivalence, although these had not been raised either by the person or by the GP during the consultation. A 42-year-old female informant said:

I felt alone with these contradictory feelings and thoughts, and my doctor did not go into it. But, of course, if I don't tell him, he doesn't get to know these feelings and reflections. It made me unsure, ..., and it reduced my desire for changing lifestyle. (id 7)

A common feature of the ambivalence sub-types was furthermore that patients seemed to shift between different conflicting feelings and reflections in a concurrent and iterative process. Thus their reflections did not always result from conflicting feelings; it was often the other way around. As a 43-year-old male informant said:

Both before and following the consultation, I was filled with conflicting feelings and multiple thoughts related to lifestyle changing and medical treatment at the same time. (id 4)

Discussion

Summary of findings

Ambivalence and its subtypes were the core motivational aspects related to potential lifestyle changes in the interviews, even if they were not verbalised during these consul-

tations. The patients perceived ambivalence as conflicting feelings about lifestyle change. These feelings interacted with their reflections in a concurrent and iterative process, ultimately making them decide whether or not to attempt lifestyle change. The analysis allowed us to generate an ambivalence typology consisting of five subtypes, each reflecting a unique dimension of the overall concept (and problem) of ambivalence: perception, demand, information, priority and treatment ambivalence.

Strengths and weaknesses of the study

The pilot study was useful for optimizing the interview guide, which was further elaborated and refined as the interviews progressed by focusing on derived analytical categories from preceding interviews. Specific questions were inspired by the videotaped consultation of each informant. The videos were made without the presence of the researcher and were not a primary data source. The patients were sampled purposefully by the GPs on the basis of specific instructions reflecting the guidelines on preventive consultations, the purpose of the study and to gather theoretical rich data. GPs were included through the health insurance register in two counties and sampled purposefully. However, some of the participating GPs may have had certain professional interests in preventive consultations. This may have shaped their choice of patients, so that they would either include rather more straightforward cases, perhaps including patients with higher than average health literacy and interest in health and lifestyle change. There was some evidence of these characteristics in the sample, but also of more problematic cases. The GPs were all aware of the guidelines related to MI but had different preventive consultation activity and competence in regarding to preventive consultations and MI. Three of the GPs had special training in MI, which may have shaped their ability to use MI and consider ambivalence for patients in the consultations. Furthermore, the non systematic use of MI must be perceived as a limitation of the results, although this is likely to reflect the reality of current clinical practice. A systematic use of MI would probably have enhanced the verbalisation of ambivalence in the consultation. Although analysis suggested 'theoretical data saturation', the sample was small and other studies with a wider range of patient types and selection are required to investigate the generalisability of the findings to preventive consultations in general. Given the concept of grounded theory and its validation as used by Strauss and Corbin [33] and referring to the constructivist position by Charmaz [34] the analysis of ambivalence and its theoretical contents was derived from the empirical material and informed by the researcher's theoretical background and interpretive understanding of the meaning of the interviews. [35] The analytical concepts and categories were, furthermore, found to be consistent with the patients' lives and statements, even though some of the quotes may present ideal answers. None of the patients had previously participated in a preventive consultation about CVD, but according to their number of risk factors and their average age over 50 years, they had probably been exposed to opportunistic preventive messages from their normal consultations, which is a limitation of the study.

The non-verbalisation of ambivalence in consultations

The frequent experience of ambivalence during the consultation makes it plausible that a preventive consultation induces ambivalence related to potential lifestyle changes among patients at increased risk of CVD. However, the patients found that GPs did not communicate systematically and purposefully about it. This could be so for several reasons. From the GP's perspective, the result could be attributed to a lack of knowledge about the frequent existence of ambivalence, a need for communicative education in handling of ambivalence or intentionally moving away from such issues in the consultation, for instance due to lack of time. Furthermore, an introduction to MI through written guidelines is inadequate to implement the

communication strategy in preventive consultations in general practice. These challenges in relation to using MI with everyday practice are important potential causes of the non-systematic use of MI, evidenced by the relative lack of implementation of MI in this study (as judged by reports from these patients). From the patients' perspective, the results may reflect their difficulties in expressing ambivalence in the consultation or unconsciousness of their ambivalence before and during the consultation, which is underlined by the fact that the patients expressed that they were much more conscious of their ambivalence in the weeks after the consultation.

The typology and informant characteristics

By analysing the ambivalence typology in the light of the informant characteristics we were not able to identify consistent relations between specific person characteristics and ambivalence subtypes. All patients experienced the different kind of ambivalences even though they had different age, gender and educational attainments.

The typology and the naming of the ambivalence subtypes

The naming of each ambivalence subtype was made on the basis of the constructivist grounded theory approach [34] and the analytical round dance [35], where the interpreting part of the analysis is open for inspiring theoretical aspects, different grounded theory methodological strategies, the researcher and participants' constructions and interpretations of the consultation and the study objectives. Other suitable labels for the ambivalence subtypes may have been found when approaching another analytical frame or strategy in data collection, preparation and analysis.

The Typology and the theoretical background

Our analysis brought forward five different ambivalence sub-types inhibiting patients' progress and preparedness to pursue lifestyle changes. These inhibitory influences are consistent with theories on the moderating effect of ambivalence in attitude-behaviour relationships, [29,30] information processing and change of attitude [2] and in particular, with the Theory of Reasoned Action (TRA) and of the Theory of Planned Behaviour (TPB) [36,37]. The TRA proposes that behaviour is predicted by intention to engage in that behaviour, which, in turn, is predicted by attitude towards that behaviour (a function of the perceived consequences of participation and a personal evaluation of those consequences) and the perceived social norm (a function of the perceived expectations to participate and the motivation to comply with those expectations). The TPB, a development of the TRA, was designed to expand the model to predict and explain behaviour that is not completely under the individual's volitional control. According to the TPB, whether someone intends to behave in a certain way depends on the extent to which that person perceives him or herself to be in control over a given behaviour, in addition to the attitudinal and social norm components included in the TRA [36,37].

At present, the non-verbalisation of ambivalence suggests that key behavioural determinants were not addressed enough in consultations, hence fundamentally undermining attempts to reduce risk behaviour. Furthermore, the sub-types can be matched to different TPB components. The perception ambivalence matches to perceived consequences, information ambivalence to personal evaluation of consequences, treatment and demand ambivalence to the perceived social norm and, finally, priority ambivalence to perceived behavioural control [37,38]. These sub-types may hence identify specific areas that can and should be addressed in consultations with the theoretical expectation and empirical backing [38] that this will enable patients to attempt and achieve lifestyle change more effectively than is currently the case.

Similarly, the different ambivalences can contribute to MI [7] and the trans-theoretical (stages of change) model of Prochaska and DiClemente [39]. The former is

based on exploring ambivalence and behaviour change through the perceived importance and perceived confidence to change behaviour [7]. The latter consists of six different stages a person goes through in his/her lifestyle changing process from the precontemplation stage to the maintenance stage. The different ambivalence sub-types can be understood as an instance of mapping to different stages of MI – perception, information and treatment map to importance, and priority and demand map to confidence. In turn, they map to the earlier and later stages in the transtheoretical model, respectively. These ambivalence subtypes hence represent specific areas that can be explored further in discussions between patients and GPs to promote lifestyle changes. However, what counts in the end, is not the GPs ability to have sophisticated discussions about ambivalence subtypes, but to improve the health profile of their patients through verbalisation of their ambivalence and increased awareness of its complexity and importance in relation to patients' motivation for lifestyle change. This can be attempted, based on the understanding gained from this study, but requires evaluation about its effectiveness for both patients and doctors.

This study did not aim to create new communication techniques, but rather to enhance existing evidence-based counselling methods in clinical practice and build on a theoretical basis for motivating and maintaining preventive health behaviour among patients at increased risk of CVD. It expands our knowledge about the central meaning of ambivalence in relation to lifestyle change and provides new insights into its complexity in the daily clinic. However, the results do not suggest that the GP should look for all the described ambivalence subtypes in the consultation to help the patient, but that the GPs and other health professionals are aware of the ambivalence phenomenon and its complexity in their motivational work with their patients. Our findings furthermore suggest that patients' ambivalence interacts with their reflections on lifestyle change in a concurrent process of managing conflicting thoughts and feelings in the consultation. This result is interesting from a cognitive psychological management perspective, because a cognitivebehavioural approach to this process of interaction aiming to change unhealthy, automated patterns of thoughts and feelings could prove instrumental in helping patients manage lifestyle change. It remains important to evaluate whether MI or other alternative cognitive strategies, enhanced by this understanding of ambivalence, could be clinically more effective in the communication about health determinants in the preventive consultations.

Conclusion

Ambivalence and its subtypes were the core motivational aspects related to potential lifestyle changes in the interviews, even if they were not verbalised during the consultations. Ambivalence and its sub-types emerged from our data collection, preparation and analysis of these interviews following preventive consultations and seemed to interact with the patients' reflections on lifestyle change in a concurrent and iterative process. This study underlines the importance of using evidenced based motivational approaches as MI, because it aims to explore and resolve ambivalence as a central motivational aspect in relation to lifestyle change.

Practice implications and future research

Future research and clinical work could explore why GPs do not talk about ambivalence in the consultations and what the consequences would be if they or other health professionals did so. Furthermore to investigate whether GPs and other health professionals' interventions to help patients verbalise their ambivalence will aid MI or other cognitive approaches thereby ensuring that patients' needs and concerns are addressed and that preventive consultations become even more effective. Finally other studies with a wider range of patient types and selection are required to investigate the generalisability of the results.

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GENERATION AND CHARACTERIZATION OF A HUMAN SINGLE-CHAIN FRAGMENT VARIABLE (scFv) ANTIBODY AGAINST CYTOSINE DEAMINASE FROM YEAST

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Background: The ability of cytosine deaminase (CD) to convert the antifungal agent 5fluorocytosine (5-FC) into one of the most potent and largely used anticancer compound such as 5-fluorouracil (5-FU) raised considerable interest in this enzyme to model gene or antibody – directed enzyme-prodrug therapy (GDEPT/ADEPT) aiming to improve the therapeutic ratio (benefit versus toxic side-effects) of cancer chemotherapy. The selection and characterization of a human monoclonal antibody in single chain fragment (scFv) format represents a powerful reagent to allow in *in vitro* and *in vivo* detection of CD expression in GDEPT/ADEPT studies.

Results: An enzymatic active recombinant CD from yeast (yCD) was expressed in E. coli system and used as antigen for biopanning approach of the large semi-synthetic ETH-2 antibody phage library. Several scFvs were isolated and specificity towards yCD was confirmed by Western blot and ELISA. Further, biochemical and functional investigations demonstrated that the binding of specific scFv with yCD did not interfere with the activity of the enzyme in converting 5-FC into 5FU.

Conclusion: The construction of libraries of recombinant antibody fragments that are displayed on the surface of filamentous phage, and the selection of phage antibodies against target antigens, have become an important biotechnological tool in generating new monoclonal antibodies for research and clinical applications. The scFvH5 generated by this method is the first human antibody which is able to detect yCD in routinary laboratory techniques without interfering with its enzymatic function.

Background

The ability of cytosine deaminase (CD) to convert the clinically used antifungal agent 5-fluorocytosine (5-FC) into one of the most potent and largely used anticancer agent such as 5-fluorouracil (5-FU) raised considerable interest in this enzyme to design innovative anticancer therapies [1,2]. Therefore, CD-based enzyme/prodrug strategies are under investigation to model gene or antibody directed enzyme-prodrug therapy (GDEPT/ADEPT) for achieving high local concentration of 5-FU without significant systemic toxicity [3,4]. In in vivo animal model, the CD gene/enzyme which is not naturally expressed in mammals are first introduced into the cells of a tumour by specific antibodies [5-7], modified microorganisms such as bacteria and viruses or synthetic vectors (reviewed by Springer et al., 2007)[4]. When the discrimination between tumor and normal tissue enzyme levels is sufficient, 5-FC is given i.v., which is converted into 5-FU by CD within the tumor [8]. A convincing demonstration that such a complex system can be developed for clinical use requires evidence that each of the components of the gene/antibody complex functions by the mechanisms proposed [9]. This can be provided by well defined measurements including the concentration levels of the antibody-enzyme conjugate or *de novo* expressed enzyme, in plasma, tumor and normal tissues [10-12]. To allow the detection of CD expression at the protein level, we raised a human monoclonal antibody in single chain fragment (scFv) format against a recombinant CD from yeast (yCD) proved to be functionally active in NMR and in *in vitro* studies to convert the antifungal drug 5-FC into the anticancer compound 5-FU. The specificity of the human scFv was confirmed by Western blot and ELISA analyses. With this antibody, yCD expression can now be

monitored without interfering with its enzymatic function in GDEPT, ADEPT and other studies leading to the effect of the so called tumour amplified protein expression and targeting (TAPET) to localize in vitro and in vivo generation of the anticancer agent 5-FU [4].

Results and discussion

The CD/5-FC-based GDEPT or ADEPT are among the most studied strategies aiming to improve the therapeutic ratio (benefit versus toxic side-effects) of cancer chemotherapy. CD has the ability to deaminate the non toxic prodrug 5-FC into the highly toxic compound 5-FU. By inhibiting DNA synthesis this drug preferentially kills tumour cells. However, 5-FU has high gastrointestinal and hematological toxicities [2]. In contrast, the prodrug 5-FC is fairly non-toxic [13]. and CD is not naturally expressed in mammalian cells. Thus, the selectively guided CD/5-FC complex should minimize the toxic effects of 5-FU because the conversion of 5-FC to 5-FU should only occur within the tumor.

A convincing demonstration that this strategy can be developed for clinical use requires knowledge of specific parameters which may include the in in vivo monitoring of the CD complex. For this reason we have firstly constructed a novel expression system for the production of a functionally active yCD. Subsequently a fully human antibody in scFv format not interfering with yCD activity was developed and analyzed.

Expression and purification of yCD protein

A functionally active yCD was generated by recombinant DNA technology. The gene encoding for yCD was amplified and inserted into the pQE30Xa expression vector which contained the *lac* promoter for protein induction and 6 Ч His TAG sequence for purification (Fig. 1A). 500 base pairs band shown in Figure 1B corresponded to DNA fragment encoding for yCD obtained by PCR using specific primers. After TG1 E. *coli* bacterial strain transformation, several clones were isolated and proved suitable for yCD production. The clone exhibiting the best protein induction was further characterized. The yield of purified protein was about 10 mg l^{-1}, using metal chelate affinity chromatography. The reliability of this novel expression system used for protein isolation and purification was confirmed by biochemical investigation showing that yCD migrated at the expected molecular weight of about 20 kDa (Figure 1C).

Selection and characterization of scFvH5 antibody specific for yCD

To isolate phage-displayed specific antibodies, an aliquot of the human synthetic ETH-2 library containing approximately 1 Ч 10^{12} cfu phages was panned into Nunc-immunotubes coated with 10 μg $ml^{-1}$of purified yCD. Nonspecifically absorbed phages were removed by intensive washing. Specific bound phages were eluted, amplified and used for next panning as previously described [14]. By using this protocol, we were able to isolate a phage-antibody population specifically recognizing yCD protein after only three rounds of selection. Plating on agar of TG1 cells infected with a pool of phage antibodies from third selection allowed individual clones harboring phagemid to grow. Soluble scFvs derived from IPTG inducted colonies, were screened by ELISA and several of them proved to be specific for yCD protein (Figure 2). One of the most reactive scFv antibody clone, named H5, was isolated and further characterized under biochemical and genetic aspects.

Western blot studies showed that scFvH5 recognizes a protein band of about 20 KDa corresponding to the expected molecular weight of the purified yCD protein (see Fig. 2, inserted box). The genes encoding for variable regions of heavy (VH) and light (VL) chains of the scFvH5 were sequenced, and their corresponding amino acid aligned (Fig. 3) according to Pini et al., [15].

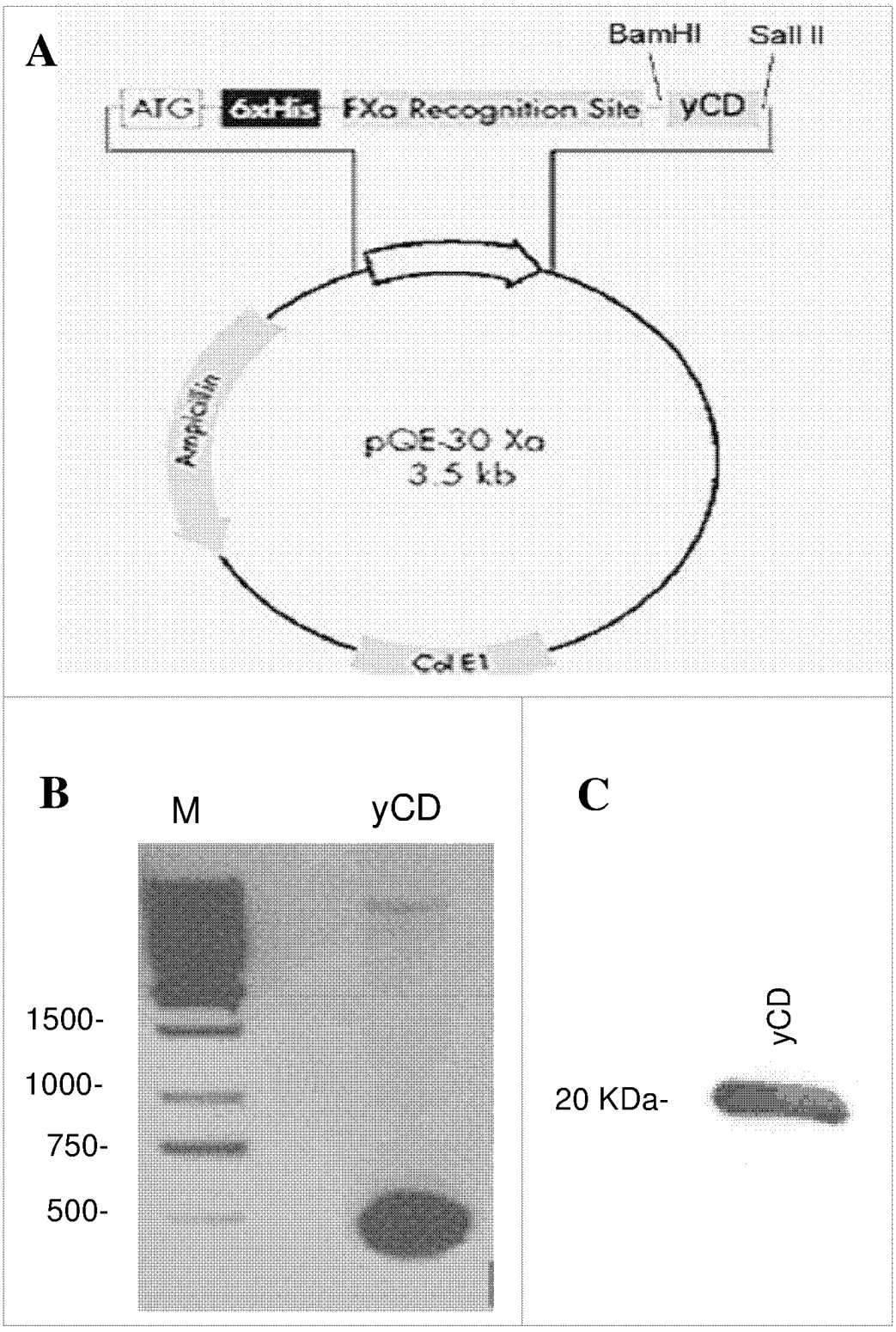

Figure 1. Expression of recombinant yCD. In (A), is depicted a schematic representation of yCD expression vector, constructed by inserting the coding sequence for yCD into pQE30Xa plasmid, and expressed in TG1 strain of *E. coli*. In (B) and (C) are shown respectively, the PCR-DNA fragment corresponding to the expected 500 bases pair encoding for yCD and the immuno-blot of the purified yCD protein.

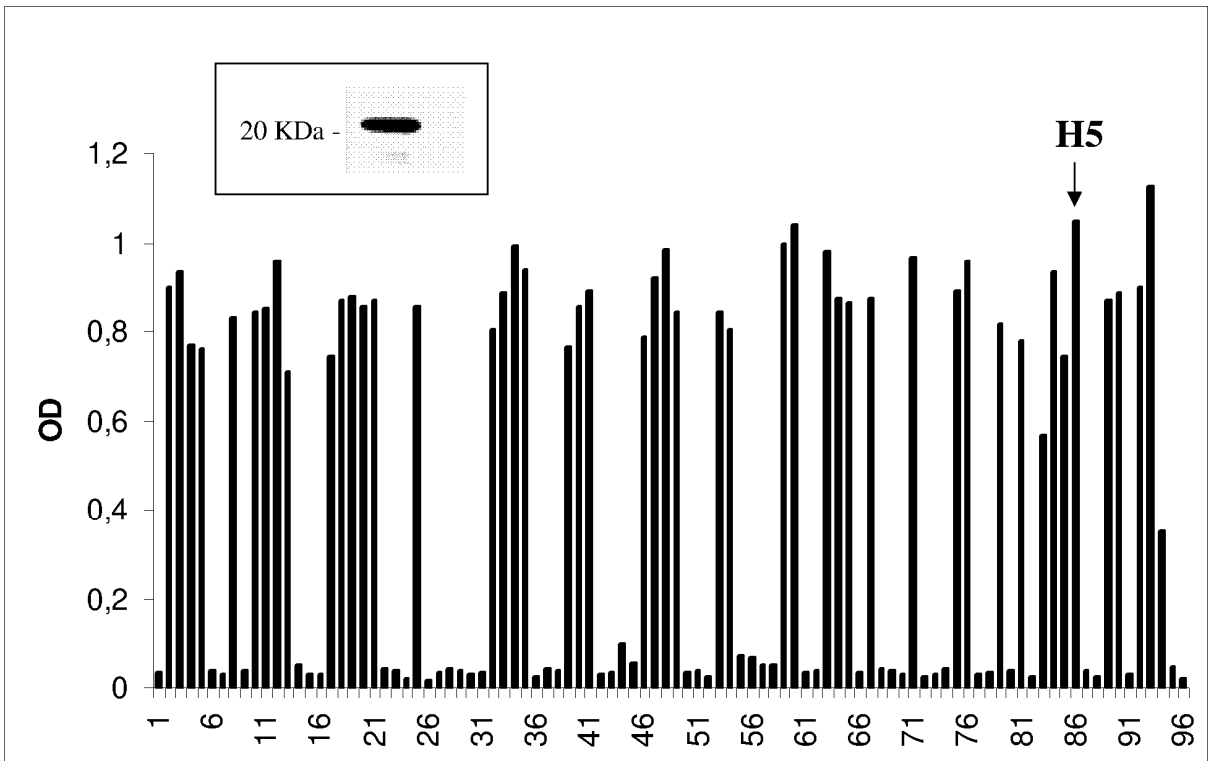

Figure 2. Selection of yCD-specific scFvs. IPTG inducted bacterial supernatants of individual colonies from the third round of the ETH-2 selection on yCD protein, were tested by ELISA in 96-well microtiter plates coated with the antigen. OD values higher than three fold the value of negative control are scored as positive. Negative and positive controls positioned in wells 1–4 reacted as expected. In the inserted box, the Western blot of yCD protein detected by scFvH5 (one of the most reactive clones) is shown.

Determination of yCD activity

In order to determine the functional activity of the recombinant yCD, the ability of the enzyme to deaminate 5-FC was assessed by fluorine NMR. This approach allowed simultaneous detection of the substrate and the product without interference by other compounds. Figure 4 shows that after 90 min 5-FC was completely converted into 5FU in the presence of the yCD. Absolute quantification of the product was obtained by adding a known amount of 5-FU to the reaction mixture at the end of the experiment.

The specific yCD enzymatic activity was also assessed by spectrophotometric analysis in order to determine nanomolar concentrations of the reaction product. Figure 5A shows the initial velocity of the reaction which is represented by direction coefficient of the line plotted placing concentration of formed 5-FU versus reaction time.

In order to assess if the enzymatic activity of yCD was affected by the presence of the scFvH5 an identical experiment was performed in presence of the antibody. Figure 5B shows that the rate of product formation was similar to that with free yCD, suggesting that there was no apparent loss in enzyme activity as a result of binding with scFvH5. Identical results were obtained using the irrelevant scFvGO antibody (see Figure 5C).

Cytotoxic assay

Using an *in vitro* model constituted by human LoVo cells, we measured the enzymatic activity of the recombinant yCD protein in converting the antifungal agent 5-FC into the highly toxic anticancer compound 5-FU. In parallel we evaluated if co-incubation of the same reagents with scFvH5 affected

yCD function. Figure 6A shows that 2.5 µg ml^{-1} of yCD exerted a significative cell growth inhibition of the human carcinoma LoVo cells in the presence of 5-FC concentration ranging from 1 mg ml^{-1} and 10 µg ml^{1}. In contrast, the co-incubation of yCD and 5-FC with various concentration of scFvH5 did not interfere with the cytotoxic activity of *de novo* generated 5-FU (Figure 6B).

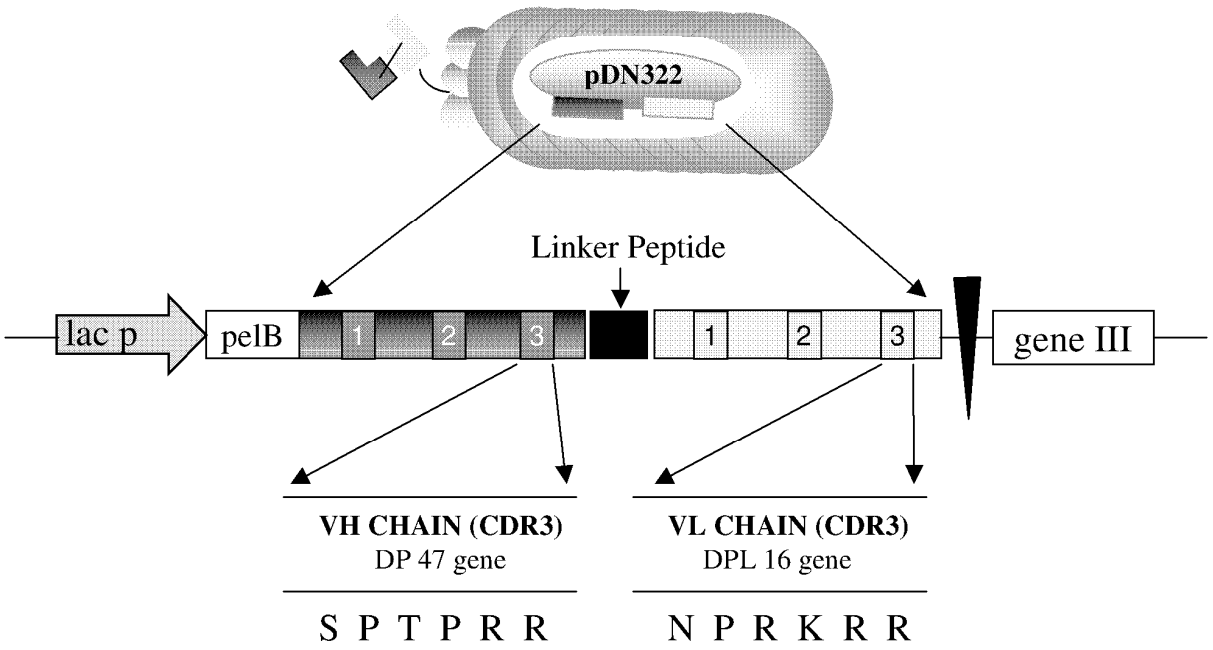

Figure 3. Sequence analysis of scFv H5 and genetic structure of phage antibody from ETH-2 library. The amino acid sequence of the CDR3 regions of the selected scFv H5 antibody are reported. A schematic representation of the scFv antibodies dislpayed on M13 phage as pIII fusion proteins is depicted.

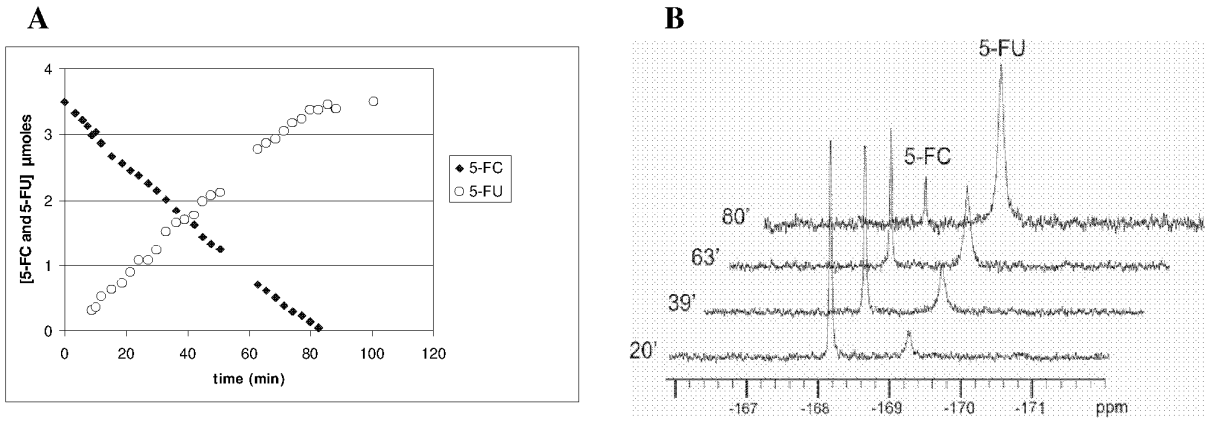

Figure 4. Functional analysis of yCD by ^{19}F NMR study. In (A) and (B) are shown respectively, the 5-FU formation (µlmoles) due to the conversion of 5-FC by yCD and representative spectra during the reaction at 20, 39, 63 and 80 min.

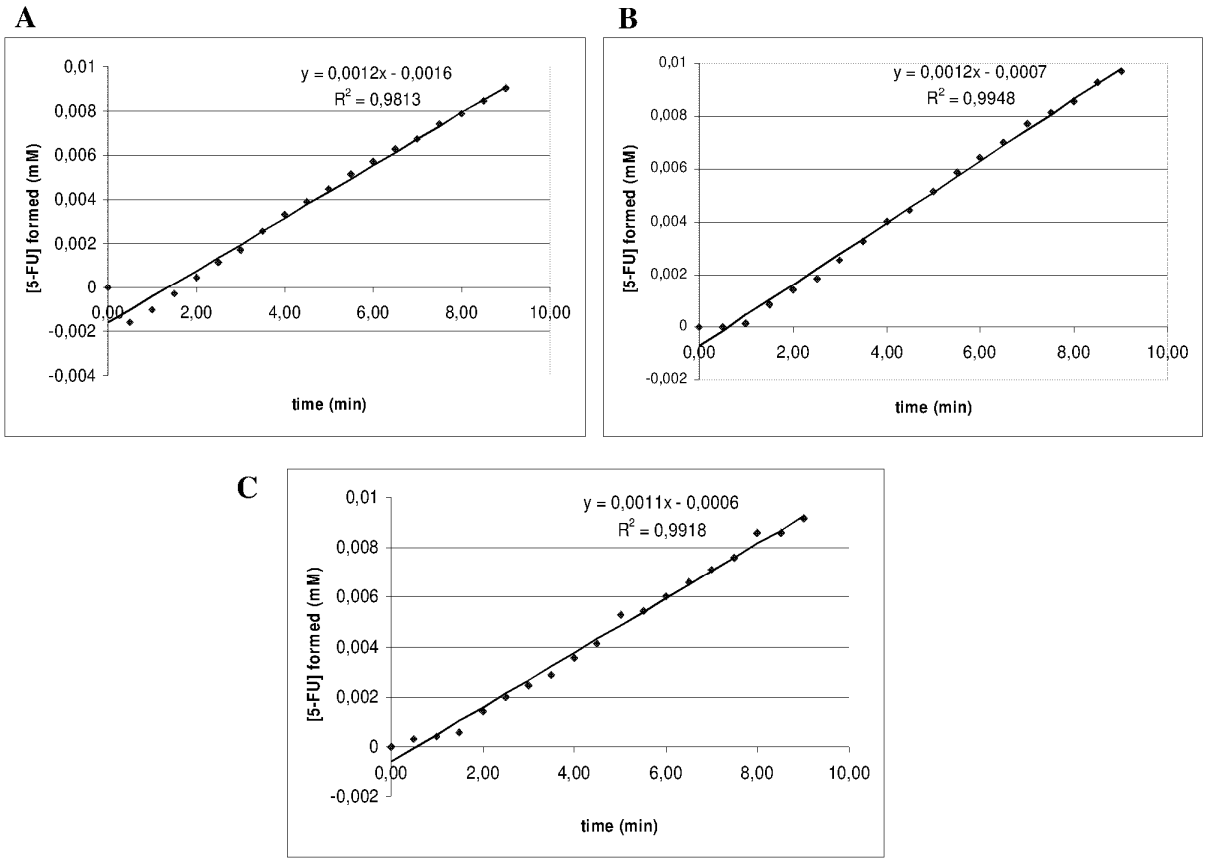

Figure 5. Spectrophotometry of yCD activity. In (A), are reported the values of de novo formed 5-FU (mM) obtained in presence of yCD (0.5 μg ml^{-1}) and 5-FC (0.18 mM) during the first 9 min of the reaction. In (B) and (C) are reported the 5-FU values obtained with identical reagents but in presence of 2 μg ml^{-1} of the specific (scFvH5) or irrelevant (scFvGO) antibodies. Slope of lines represents starting speed of the reaction. Correlation coefficient (R) indicates the strength and direction of the linear relationship between time and formed 5-FU.

The results above reported demonstrated that, yCD produced by the novel expression system here described acts as an active enzyme in converting 5-FC into the anticancer compound 5-FU. Moreover, the binding of the human scFvH5 with yCD did not affect the enzyme function. In particular, our studies demonstrated that the presence of scFvH5 did not interfere with yCD in converting 5-FC or with the cytotoxic activity of *de novo* formed 5-FU.

Conclusion

The monoclonal antibody scFvH5 may be a very useful reagent for detection of CD expression in GDEPT/ADEPT studies. In fact, this mAb detects functional yCD either in ELISA or in Western blot studies (Figure 1 and 2) thus providing evidence that similar techniques may be extended to measure yCD levels in plasma, tumor and normal tissue samples. Since its particular genetic origin, the scFvH5 can be easily genetically engineered to construct a whole human antibody with a predefined IgG subclass, for selective removal of mAb-yCD conjugate from the circulation, without interfering with the enzyme function.

Differently with other mAbs to CD generated by hybridoma [5] or recombinant DNA technologies [16], the scFvH5 is the first fully human monoclonal antibody in scFv format so far described which is able to detect yCD protein in different routinary laboratory techniques. Hence, this antibody

may represents an excellent candidate for in vivo detection and measurement of the CD complex in the future development of CD-based selectively guided tumor therapy.

Methods

Antibodies and reagents

The characteristics of the scFvGO used in this study as scFv irrelevant antibody were previously described [17]. AntiFlag M2 and anti-polyhistidine antibodies were purchased from Sigma (St Louis, MO, USA). The goat antimouse HRP-conjugated polyclonal antibody was purchased from Dako (Denmark, EU). 5-Fluorocytosine (5FC) and 5-Fluorouracil (5-FU) were purchased respectively, from Sigma and Mayne Pharma (Naples, Italy, EU).

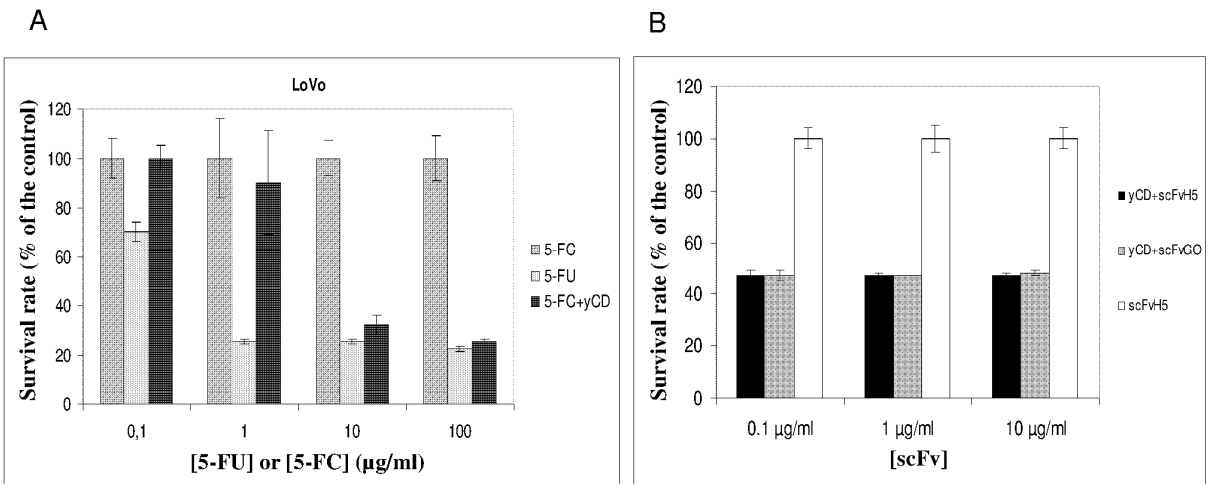

Figure 6. In *in vivo* assay of yCD protein. In (A), LoVo cells were seeded in 96-well plate (2500 cells/well) and cultured in BM for 4 days containing 2.5 µg ml^{-1} of yCD in presence of the indicated concentrations of 5-FC. In (B), the cells were culture at same conditions but in BM containing 2.5 µg ml^{-1} of yCD and 10 µg ml^{-1} of 5-FC in presence of different concentrations of scFvH5 or the irrelevant scFvGO antibodies. Cell cytotoxicity (due to *de novo* formed 5-FU) was evaluated by WST-1 assay and calculated as a percentage of survived cells. Values are reported as the mean of triplicate samples. The bars indicate SD.

Vector construction

Complete yCD gene sequence [18] was amplified by PCR from cDNA inserted in pACCMV 115. The sense primer was: *BamyCD* 5'-CGA ATT GGA TCC ATG GTG ACA GGG GGA-3', containing BamHI restriction site and the sequence coding for first five amino acid of yCD. The antisense primer was: *ESyCD* 5'-ATCC GAT ATC GTC GAC CTC ACC AAT ATC TTC-3' containing the sequences encoding for the end part of yCD and SalI restriction enzyme.

PCR was performed using Pwo enzyme (Roche Diagnostics; IN, USA) and the resulting PCR fragment was agarose-purified using the High Pure PCR Product Purification Kit (Roche). Then it was digested with restriction enzymes *BamHI* and *SalI*, and cloned into the plasmid pQE30Xa (Qiagen; Milan, Italy, EU), containing 6 Ч His tag sequence for protein purification. The clone was sequenced by Biofab Research SRL (Rome, Italy, EU).

Expression and purification

TG1 *E. coli* (*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F' [*traD*36 *proAB*+ *lacIqlacZ*ΔM15]) cells trasformed with plasmid pQE30Xa yCD were grown in 100 ml 2 Ч TY broth supplemented with 100 µg ml^{-1} ampicillin and 0.1% glucose in a 37°C shaker until OD_{600} = 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) was added to a final concentration of 1 mM. Cells were harvested 3 h later, centrifuged at 10,000 rpm for 20 min at 4°C and

lysed by sonication in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8). The yCD protein was purified by affinity chromatography on Ni-NTA resin (Qiagen), using native protocol according to the manufacture instructions. Protein concentration was determined with Fernandez-Patron method. The purified yCD protein was dissolved in PBS, aliquoted and stored at - 80°C.

NMR

^{19}F NMR analyses were performed on BRUKER AVANCE spectrometer (Bruker BioSpin GmbH – Rheinstetten – Germany) operating at 9.4 T. The spectra were acquired at 25°C with a pulse angle of 60°, interpulse delay of 2 s and 64 transients. In order to compensate for partial magnetic saturation effect, the correction factors were determined by comparing the measured peak areas with those obtained at equilibrium (flip angle 90°, interpulse delay 30 s). At the end of reaction the concentration of 5-FU was determined by adding a known amount of the drug. Spectral analyses were performed utilizing the XWIN-NMR BRUKER suite. ^{19}F-MRS of 3,5 µmoles of 5-FC dissolved in 700 ul D_2O saline buffer was considered the time 0 of the reaction and after 70 µl of 25 µg/ml yCD enzyme were added. The reaction was followed during 1 h and 30 min. To verify the complete conversion of 5-FC to 5-FU the last spectrum was acquired at 3 h and 15 min.

ETH-2 library

The ETH-2 synthetic human recombinant antibodies library consists of a large array (more than 10^9 antibody combination) of scFv polypeptides displayed on the surface of M13 phage [14]. It was built by random mutagenesis of the CDR3 of only three antibody germline gene segments (DP47 for the heavy chain, DPK22 and DPL16 for the light chain). Diversity of the heavy chain was created by randomizing four to six position, replacing the pre-existing position 95–98 of the CDR3. The diversity of the light chain was created by randomizing six position (96–101) in the CDR3 [15].

Selection of yCD protein specific antibodies from ETH-2 library

Immunotubes (Nalge Nunc International; NY, US) were coated overnight (ON) at room temperature (RT) with purified yCD in PBS at the concentration of 10 µg ml^{-1}. After panning, performed according to Ascione et al. [17], phages were eluted with 1 ml of 100 mM triethylamine, and the solution was immediately neutralized by adding 0.5 ml of 1 M Tris-HCl pH 7.4. Eluted phages were used to infect TG1 *E. Coli* cells and amplified for the next round of selection. Briefly, 50 ml of 2 Ч TY with 100 µg/ml ampicillin and 1% glucose (2 Ч TY-amp-glu) were inoculated with enough bacterial suspension to yield an $OD_{600\ nm} \cong 0.1$. The culture was grown to $OD_{600\ nm} = 0.4–0.5$ and infected with K07 helper phage at a ratio of around 20:1 phage/bacteria. The rescued phages were concentrated by precipitation with PEG 6000 and used for the next round of panning. For soluble scFv preparation, cloned E. coli cells were grown for 2 h at 37°C in 180 µl of 2 Ч TY-ampicillin (100 µg ml^{-1}) and 0.1% glucose in 96-well plates and induced with 50 µl of 2 Ч TY-6 mM IPTG. The following day the plates were spun down at 1800 g for 10 min at 4°C and the supernatants containing soluble scFv were recovered and tested for specific yCD recognition in ELISA.

ELISA

96-well ELISA plates were coated ON with 50 µl/well of 10 µg ml^{-1} purified yCD in PBS at 4°C. Next day a blocking solution, 2% non-fat milk in PBS (2% MPBS) was added and after 2 h the plates were washed with PBS containing 0.05% Tween 20 (TPBS). Plates were incubated for 2 h at RT with 50 µl of supernatants containing soluble scFv antibodies, anti-Flag M2 antibody and anti-mouse HRP-conjugated antibody. All antibodies were resuspended in 2% MPBS.

The reaction was developed using 3,3'-5,5'-tetramethylbenzidin BM blue and POD substrate soluble (Roche Diagnostics) and stopped by adding 50 µl of 1 M sulfidric acid. The reaction was detected with an

ELISA reader (BIORAD; CA, USA) and the results were expressed as OD, i.e. the absorbance per unit length, were absorbance (A) is calculated as A = A (450 nm) – A (620 nm).

DNA characterization and sequences

Plsmidic DNA encoding for selected scFvs were digested by specific endonucleases and CDR3 regions were sequenced with an automated DNA sequencer (M-Medical/Genenco, Pomezia Italy) using fdseq1 (5'-GAA TTT TCT GTA TGA GG-3') and pelBback (5'-AGC CGC TGG ATT GTT ATT AC-3') primers.

Soluble scFv purification

The clone scFvH5, was cultured for large-scale scFv production. TG1 *E. coli* infected cells were cultured at 30°C in 2 Ч TY containing 100 μg ml^{-1} ampicillin and 0.1% glucose up to OD_{600} = 0.5. After induction of antibody expression by adding 1 mM IPTG to culture, cells were incubated ON at 30°C. Then, the bacterial culture was centrifugated and antibody containing supernatant collected. Antibody fragments were precipitated with ammonium sulfate and dialyzed in PBS. His-tagged scFv fragments were purified by immobilized metal affinity chromatography using Ni^{2+}-nitriloacetic acid agarose (Qiagen). ScFv fragments were eluted with 250 mM imidazole in PBS, dialyzed, ELISA tested for specific antigen recognition, and stored at - 80°C.

SDS-PAGE and Western Blot analysis

Purified yCD protein was analyzed on 12% SDS PAGE gel under reducing conditions. Gel was either stained with Fernandez-Patron method or blotted electrophoretically to nitrocellulose membrane, which was blocked in 5% MPBS and then washed three times for 10 min in PBS. For detection of yCD protein, the membrane was incubated either with anti-polyhistidine antibody or with soluble scFvH5. In the first case the membrane was incubated for 2 h with anti-polyhistidine antibody 1:1000 in 2% M/PBS and washed three times with PBS. In the other, the membrane was incubated for 2 h with soluble scFvs, washed with PBS containing 0.05% Tween 20 and incubated again with an anti-Flag M2 mouse antibody 1:1000 in 2% MPBS for 1 h at RT. In both cases specific binding was detected by HRP-conjugated Goat anti-mouse antibody 1:1000 in M/PBS 2% for l h at RT. After 3 washings in 2% M/PBS, the bound antibodies were visualized with DAB buffer obtained by dissolving one tabelet (10 mg) of 3,3'diaminobenzidine (Sigma) in 20 ml of PBS and 3 μl of hydrogen peroxide 30%, for 3 min. The reaction was stopped with H_2O.

Determination of yCD activity

The deamination activity of purified yCD was measured by monitoring conversion of 5-FC to 5-FU in spectrophotometric studies. In 0.5 ml quartz cuvette, 250 **o**l of 1 **n**g ml^{-1} yCD was added to solution of 0.36 mM of 5-FC. The reaction was followed for 30 min by an UV/Vis spectrophotometer (Beckman DU-64, Beckman Coulter S.p.A., CA, USA) which registered absorbance values every 30 seconds. The absorbance variation was measured at 265 nm, wavelength of the 5-FU maximum UV absorption according to Nishiyama et al., 1985 [17]. Absorbance values were calculated as A_{265} (t) A_{265} (t_0), (t_0 = 0 min); the values were converted in concentration of formed 5-FU, dividing absorbance values by 5-FU molar extinction coefficient at 265 nm (s_{265}). The calculated 5-FU s_{265} was 7 mM^{-1} cm^{1}. Initial velocity of the enzyme was calculated as AA_{265} min^{-1} or as A[5-FU] min^{-1} in the first 9 min when the reaction had linear trend.

The same procedures were used in order to examine eventual inhibition of yCD activity occurred in presence of scFvH5. Briefly, 5 |**J**I of 200 ***ig** ml^{-1} purified scFvH5 solution were added into the cuvette with yCD and 5-FC. Parallel experiments were performed in presence of the irrilevant scFvGO antibody.

Cytotoxic assay

The ability of purified yCD protein to convert 5-FC into 5FU was tested in an vitro cell sytem. The human colon adenocarcinoma LoVo cells were maintained in a basic

medium (BM) constituted by RPMI 1640 (EuroClone S.p.A; PV, Italy, EU) supplemented with 10% fetal bovine serum (EuroClone) and 1% penicillin-streptomycin in humidified atmosphere with 5% CO_2 at 37°C.

In a cell growth inhibition assay 2500 cells/well were seeded into 96-well microtiter plates (Corning Cable Systems SRL, Turin, Italy, EU) in BM containing 2.5 \ig ml^{-1} of yCD and different concentrations of 5-FC. The plates were incubated at 37°C for 4 days and cell viability was evaluated by WST-1 assay (Takara, VinciBiochem, Vinci, Florence, Italy, EU).

As positive and negative controls different concentrations of 5-FC and 5-FU alone were used in identical in vitro conditions. A cell growth inhibition assay was also used in order to determine whether the binding with the specific scFvH5 antibody affects yCD enzyme function.

In this experiment LoVo cells (2500 cells/well) were seeded in 96-costar plates in BM containing 2.5 \ig ml^{-1} of yCD and 10 |ig ml^{-1} of 5-FC in presence of scFvH5 or scFvGO antibodies at concentrations ranging from 0.1 to 10 |ig ml^{-1}. All results were represented as the mean of triplicate samples.

Abbreviations

5-FC: 5-fluorocytosine; 5-FU: 5-fluorouracil; yCD: yeast cytosine deaminase; scFv: single chain fragment variable; GO: glucose oxidase; mAb; monoclonal antibody

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ALLOGENEIC AND AUTOGENOUS TRANSPLANTATIONS OF MSCs IN TREATMENT OF THE PHYSEAL BONE BRIDGE IN RABBITS

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Background: The aim of this experimental study on New Zealand's white rabbits was to find differences in the results of treating the distal physeal femoral defect by the transplantation of autologous or allogeneic mesenchymal stem cells (MSCs). After the excision of a created bone bridge in the distal physis of the right femur, modified composite scaffold with MSCs was transplanted into the defect. In animal Group A (n = 11) autogenous MSCs were implanted; in animal Group B (n = 15) allogeneic MSCs were implanted. An iatrogenic physeal defect of the left femur of each animal not treated by MSCs transplantation served as control. The rabbits were euthanized four months after the transplantation. The treatment results were evaluated morphometrically (femoral length and valgus deformity measurement) and histologically (character and quality of the new cartilage).

Results: Four months after the transplantation, the right femurs of the animals in Group A were on average longer by 0.50 ± 0.04 cm (p = 0.018) than their left femurs, the right femurs of rabbits in Group B were on average longer by 0.43 ± 0.01 cm (p = 0.028) than their left femurs.

4 months after the therapeutic transplantation of MSCs valgus deformity of the distal part of the right femur of animals in Group A was significantly lower (by 4.45 ± 1.86°) than that of their left femur (p = 0.028), in Group B as well (by 3.66 ± 0.95° than that of their left femur p = 0.001). However, no significant difference was found between rabbits with transplanted autogenous MSCs (Group A) and rabbits with transplanted allogeneic MSCs (Group B) either in the femur length (p = 0.495), or in its valgus deformity (p = 0.1597). After the MSCs transplantation the presence of a newly formed hyaline cartilage was demonstrated histologically in all the animals (both groups). The

ability of transplanted MSCs to survive in the damaged physis was demonstrated in vivo by magnetic resonance, in vitro by Perls reaction and immunofluorescence.

Conclusion: The transplantation of both autogenous and allogeneic MSCs into a defect of the growth plate appears as an effective method of surgical treatment of physeal cartilage injury. However, the Findings point to the conclusion that there is no clear difference in the final effect of the transplantation procedure used.

Background

By impairment of enchondral ossification and normal chondrogenesis in the area of growth cartilage of the long bones of the extremities, a formation of the bone bridge may occur with a subsequent disturbance of bone growth [1,2]. For a number of years, rooted methods of treatment of the physeal plate closure due to its trauma have been passed on [3-5]. However, considering their exigence of time and finances and the seriousness of possible complications, different procedures have been searched for in experiments, that would allow correcting deformities (or even to prevent the development of such deformities) of the long bones of the extremities with an injured physis less invasively and with a better clinical effect and lower percentage of

complications. Some departments have started to focus their attention on transplantations of tissues and cellular colonies into the defect with the aim to restore physeal morphology and function. In the past six years we have pursued experiments with the transplantation of chondrocytes and mesenchymal stem cells (MSCs) into the damaged growth plate with the purpose to restore its function (transplantation of autologous chondrocytes into an iatrogenically injured growth plate of the distal femur of the pig, prevention of formation of a bone bridge by transplantation of allogeneic MSCs into an iatrogenically created defect, therapy of a formed bone bridge by its excision and transplantation of allogeneic stem cells into the created defect) [6-9]. Yet, in the area of cellular transplantation a number of questions remain unanswered before their possible clinical use in humans can happen. One of these questions is also the aspects of autogenous vs. allogeneic transplantations of mesenchymal stem cells into the injured physeal growth zone of the extremity bones.

The study of possible repair of the growth cartilage tissue has notably benefited and advanced thanks to works addressing the transplantation of autogenous chondrocytes into the growth cartilage defect [10-14]. In practice, transplantation of autogenous chondrocytes represents the collection of the cartilaginous tissue and subsequently the cultivation of a chondrocyte graft that may be used for the autogenous transplantation into a growth cartilage defect after the lapse of approximately 3 weeks [15]. This time lag between the autograft sampling and on possibility to implant cultivated cells might limit the autotransplantation in actual clinical practice. Therefore, allograft transplantation appears suitable in this regard, offering, provided the availability of a tissue bank, the possibility of immediate cell transplantation into the damaged target tissue.

The aim of this experimental study focusing on the surgical treatment of an iatrogenically created bone bridge in the distal physis of the femur in rabbits in form of its excision and subsequent transplantation of MSCs into the physeal defect, was to find whether there would be differences between the results of treatment by transplantation of autologous mesenchymal stem cells and by transplantation of allogeneic MSCs, always in the same gel scaffold. We evaluated the results of treatment partly on the basis of measurements of the length and valgus deformity of the femurs (morphometric variables of bone growth) and partly with regard to the quality of the newly formed cartilaginous tissue at the site of the original physeal defect (histological examination).

The proof that the newly formed chondrocytes at the site of the original physeal defect originated from the transplanted mesenchymal stem cells was established partly in vivo by demonstrating cells labelled with ferrous nanoparticles in the magnetic resonance examination and partly in vitro using Pearls reaction and immunofluorescence.

Methods

The New Zealand white rabbit from a certified breeding was chosen as the experimental animal. The experiment included 26 healthy animals (12 males and 14 females) at the age of 5 weeks at the bone marrow collection (at the beginning of the experiment). The group of animals was homogenous as regards weight (2.32 ± 0.14 kg). The rabbits were divided into two groups: Group A (therapeutic autogenous transplantation of MSCs into the growth cartilage defect – 13 animals); and Group B (therapeutic allogeneic transplantation of MSCs into the growth cartilage defect – 13 animals). However, due to difficulties with MSC cultivation (the autogenous graft did not reach the needed number of cells on the day of transplantation) two experimental animals from Group A had to be transferred

to Group B. Thus, the final number of animals evaluated in Group A was 11 and the final number of animals in Group B was 15.

Preparation of the stem cells and the scaffold

Twenty one days before the transplantation of MSCs bone marrow blood was col-

lected in general anaesthesia from the iliac wing on both sides. Using a punction needle (20 G/40 mm) an average of 2 ml blood was aspired from *tuber coxae alae osis illi* in to the 5 mL syringe with 2 mL Dulbecco's Phosphate Buffered Saline (PBS) with 2% Fetal Bovine Serum (FBS, StemCell Technologies) and 5 IU heparin/mL connected with a hypodermic needle (20 G/40 mm). Under sterile conditions, the bone marrow blood (about 4 mL) was deposited over 3 mL of FicollPaque PLUS (StemCell Technologies). After centrifugation at 400 g for 30 min at room temperature, the dense gradient separated erythrocytes and granulocytes as a pellet in a bottom part of the tube; mononuclear cells were situated in an opalescent layer between Ficoll and blood plasma. This layer was taken out, washed in a culture medium (see below) and used for propagation under in vitro conditions. The average amount of mononuclear cells from each isolation was 20 X 10^6 cells. Cell number and viability was analyzed on Vi-CELL (Series Cell Viability Analyzers) and about 90% of viable cells were detected.

Cells were seeded in 75-cm2 tissue culture plastic flasks at a density of approximately 5 X 10^5 cells/cm2 and cultured at 37°C in humidified atmosphere with 5% of CO2. The culture medium was a-MEM medium (Gibco) supplemented with 10% FBS (Sigma Aldrich) and gentamycin (50 mg/mL, Sigma Aldrich). After 24 h of culture, the nonadherent cells were removed and during the subsequent culture (3 weeks) the medium was exchanged every third day. The first colonies of mesenchymal stem cells appeared after 4 to 5 days of culture and the 80% of confluence was achieved after 10 days of culture. Cells were passaged with trypsin 0.5% trypsin-EDTA solution (Sigma Aldrich) for 5 min at 37°C and replated in 150 cm2 at a density of 5000-6000 cells/cm^2.

During the last three days of culture, cells were labeled with nano-particles of iron oxide (Resovist, 0.5 mmol Fe/ mL, Schering). Resovist was added in concentration 1 ***iL**/ mL culture medium. For labeling with a fluorescent dye CM-DiI (Molecular Probes) in concentration 5 ng/2,5 mL PBS, cells were harvested on day of transplantation, incubated for 5 min at 3 7°C and for 15 min at 4°C. At the end of labeling cells were thoroughly washed in PBS. To induce chondrogenic differentiation [15], the labeled cells were given in the differentiation medium composed of **a**MEM supplemented with 100 ng/mL recombinant human TGF **(3**1 (R&D Systems), 100 nM dexamethasone (Medochemie), 50 µg/ml L-ascorbic acid 2-phosphate (Sigma Aldrich), 1% insulin-transferrin-selenium A (Gibco) for 30 min. Subsequently, the cells were centrifuged at 700 g for 5 min, and cell pellets were prepared for their deposition in a scaffold.

The scaffolds were prepared in a 96 – well plate at about 4°C by mixing 20,75 µL of sodium hyaluronate (10 mg/ mL, 1500 kDa, kindly provided by CPN, CR) with 31,1 µL of 1 mg/mL type I collagen in 0.1 M acetic acid (Collagen type I from calf skin, acid soluble, Sigma), and neutralized with 1 M KOH. Then a cell pellet (2 X 106 cells), resuspended in 36 µL of α-MEM was added. Subsequently, 120 µL of Tissucol solution in aprotinine (fibrinogen 70–110 mg/mL, aprotinine 3000 KIU/mL), and 120 µL of thrombin solution (4 IU/mL) in CaCl2 (40 µmol/mL, Tissucol® Kit, Baxter) were mixed and added to each well and a content of each well was thoroughly mixed. The gel was formed at 37°C for 15 min. Subsequently, the culture medium was added and the scaffold was placed in an incubator with a humidified atmosphere, 5% CO2 at 37°C and implanted on the same day.

Surgical procedures

Surgeries were performed under general anaesthesia. Before anaesthesia, enrofloxacin (BAYTRIL 2.5% inj. ad us. vet., Bayer) was administered intravenously at the dose of 5 mg/kg. Induction was achieved by intramuscular administration of midazolam (1.00 mg/kg (DORMICUM inj., Roche) + fentanyl (0.02 mg/kg (FENTANYL, Janssen) and medetomidine at the dose of 200 µg/kg (DOMITOR inj. a.u.v., Pfizer). Total inhala-

tion anaesthesia was then maintained by a mixture of oxygen, nitrous oxide (2 : 3) and isoflurane (FORANE, Abbott Laboratoires) using a non re-breathing system (Bain). The heart rate, respiratory rate, invasive blood pressure, end-tidal partial pressure of carbon dioxide and saturation of haemoglobin by oxygen was monitored (DATEX Cardiocap II). As this combination of drugs causes a strong respiratory depression, all animals were connected and controlled by ventilation device.

Rabbits were placed in dorsal recumbency and the surgical site was routinely prepared for the aseptic procedure on both knees. Lateral arthrotomy of the stifle joint was performed by parapatellar incision. After visual localization of the growth plate, the battery-powered drill (Colibri system, SYNTHES, USA) was used to create a defect in the lateral part of the distal femoral physis in order to cause damage exceeding 9% of the growth plate area [5,13,14]. Therefore, a 3.5 mm drill bit (ACUFEX – MosaicPlasty Precision, Smith&Nephew, USA) was used to bore a canal 12 mm deep from the lateral surface of the lateral condyle dorsolaterally above the insertion of m. extensor digitorum longus. The canal was drilled in the dorsomedial direction in order to cause damage of the lateral part of the distal femoral physis including the adjacent parts of epiphysis and metaphysis. The external part of the canal of the iatrogenic defect of the distal physis of the right femur was closed only with a cylinder made from beta-tricalcium phosphate (ChronOS, SYNTHES) 3.5 mm thick and 2 mm long, that was cut out from a pre-formed ChronOS block using a 3.5 mm tubular chisel (ACUFEX – MosaicPlasty Precision, Smith&Nephew, USA). This bioceramic cylinder was stained with methylene blue (ModR metylenovA, ind., 100 g, FISHER SCIENTIFIC) for easier orientation during the following arthrotomy with the bone bridge excision and therapeutic transplantation of MSCs.

The stifle joint was lavaged with Ringer Lactat solution (Ringer Lactat I.V.Inf., Braun Medical AG). The joint capsule was closed with an interrupted suture (polypropylene, Prolene 4/0, Ethicon). The subcutaneous layer was closed with a continuous suture using 2/0 polyglactin 910 (Vicryl, Ethicon). The skin was closed with a simple interrupted suture using 2/0 polyglactin 910 (Vicryl, Ethicon).

This iatrogenically created defect of the distal physis of the right femur in animals of Groups A and B then served as the site of transplantation of the gel scaffold with autogenous mesenchymal stem cells (Group A) or allogeneic MSCs (Group B). The defect of the growth cartilage was created using the same method in the left femoral bone and was left in all the animals in groups A and B without transplantation of MSCs and served in both groups as control.

Three weeks after causing the damage we performed arthrotomy of the right knee joint using the same method in animals of both groups A and B. The bioceramic cylinder stained with methylene blue and the bone bridge formed at the site of the original physeal defect were bored off by a drill with a 3.5 mm diameter (SYNTHES). Before transplantation of the scaffold with MSCs, the canal was dilated using a 3.5 mm dilator (ACUFEX – MosaicPlasty Precision, Smith&Nephew, USA). A mixture of the scaffold and MSCs (autogenous in Group A; allogeneic in Group B, respectively) was prepared in wells of a microtitration plate (TPT), from where the implant (in the form of a cylinder 3.5 mm thick and 10 mm long) was taken by the drill guide (ACUFEX – MosaicPlasty Precision, Smith&Nephew, USA) and carefully inserted using a delivery tamp (ACUFEX – MosaicPlasty Precision, Smith&Nephew, USA) into the defect drilled in the lateral right femoral condyle. In order to fix the transplant in its position, the canal was closed (on the lateral surface of the lateral condyle of the femur) with a cylinder made from beta-tricalcium phosphate (ChronOS, SYNTHES) 3.5 mm thick and 2 mm long, that was cut out from a pre-formed ChronOS block using a 3.5 mm tubular

chisel (ACUFEX – MosaicPlasty Precision, Smith&Nephew, USA).

Antagonization of all three anaesthetic components was performed using a combination of naloxon (0.03 mg/kg) (INTRENON inj., Leciva a.s.) + flumazenil (0.1 mg/kg) (ANEXATE, Hoffmann-La Roche Ltd.) + atipamezol (1.0 mg/kg) (ANTISEDAN inj. ad us. vet., Pfizer Animal Health) administered intramuscularly after the surgery. Analgesia in the post-operative period was achieved by administration of carprofen (RIMADYL inj. ad us. vet., Pfizer Animal Health) at the dose of 2 mg/kg/day for three days after the surgery. The animals were allowed to walk freely and bear weight as tolerated following recovery from surgery. The animals were fed, handled and housed according to the principles of welfare during the whole study period. At the end of the experiment (4 months after the first surgery), all animals were euthanized lege artis. Firstly, they were put under general anaesthesia using intravenous thiopental at the dose of 20 mg/kg; then they were given intravenous T 61 inj. ad us. vet. (Hoechst Roussel Vet.) at the dose of 1 ml pro toto.

The length and angular (valgus) deformity of the operated bone were measured from radiographs in the craniocaudal (CC) projection. The quality of graft incorporation was evaluated histologically. The presence of transplanted cells in the physis was detected by immunofluorescence. All procedures were conducted with the consent of the Ethical Committee (No. 46613/2003-1020).

Bone length discrepancy and femoral valgus deformity measurements

Each rabbit was subjected to radiological examination on the day of the first surgery (bone marrow blood harvesting), and after euthanasia. Bone length discrepancy and valgus deformity were measured from radiographs. Length measurement of the right femur (with the physeal defect and transplanted MSCs) and the left femur (with the physeal defect without transplanted MSCs) was done from radiographs of the femur in the craniocaudal (CC) projection. Actual length of the femur and the angle of valgus deformity of the distal femur were measured. The measurements were performed separately by three independent observers. The measured values were averaged to calculate the arithmetic mean.

Magnetic resonance imaging

We used magnetic resonance imaging to in vivo detection of transplanted MSCs in the physeal defect. The rabbits were subjected to MRI examination three weeks after the surgery and on the day of euthanasia – they were examined by the technique of T1 weighed images and by the sequence modified to highlight the hyposignal of MSCs labelled with iron oxide (detection of paramagnetic iron oxide nanoparticles, Resovist). A three-week interval between the transplantation and the first MRI examination was allowed to eliminate possible formation of artifacts caused by postoperative haematoma [16].

Histological findings

The defect healing was examined histologically using haematoxiline and eosin staining. Following the excision of femurs of the euthanized rabbits, femoral distal epiphyses were placed and stabilized in a 10% solution of formalin. They were then decalcified and gradually dehydrated in solutions with an increasing concentration of alcohol to enable them take to paraffin. Ultrathin paraffin sections of the distal femur 0.1 mm in thickness were stained with haematoxiline and eosin (HE) and subjected to microscopy, histochemical analysis – collagen – 2 immunostaining (Picture 4) and PAS reaction were provided. The defect site in the growth plate of the femur was examined histologically. The presence of the hyaline cartilage in the original defect was observed, as well as possible histological signs of transplant rejection (lymphocyte infiltrate, cartilage separation from the surrounding tissue, ligament degeneration). The ferrous stain Resovist incorporated in the MSC cytoplasma allowed the processing of one of the histological sections for Pearls reaction (staining with Berlin blue)

and thus to verify the origin of chondrocytes from the transplanted MSCs.

These examinations should prove whether the chondrocytes present in the defect originated from the implanted colony of MSCs or not on the basis of immunofluorescence detection of the CM-DiI stain incorporated into the cell wall.

Statistical evaluation

Means and standard deviations were calculated for the length and valgus deformity of the right femur (with the physeal defect and transplanted MSCs) and the left femur (with the physeal defect without transplanted MSCs) as well as for differences in length and angular deformities before MSCs transplantation and after euthanasia. The values were statistically analyzed using Wilcoxon matched-pairs test; STATISTICA (data analysis software system), version 7.1 (StatSoft, Inc. 2005).

Results

No animal suffered perioperative complications or premature death. During the cultivation of MSCs, two experimental animals had to be transferred from Group A to Group B, as their autogenous graft did not reach the number of cells needed for a successful transplantation. Four months after the transplantation, the right femurs of animals of Group A (with the excised bone bridge and transplanted autogenous MSCs) were longer on average by 0.50 ± 0.04 cm ($p = 0.018$) than their left (control) femurs (without transplanted MSCs). 4 months after the transplantation the right femurs of rabbits of Group B (with the excised bone bridge and transplanted allogeneic MSCs) were longer on average by 0.43 ± 0.01 cm ($p = 0.028$) than their left (control) femurs (without transplanted MSCs).

4 months after the therapeutic transplantation of MSCs was the valgus deformity of the distal part of the right femur in the animals of Group A (with the excised bone bridge and transplanted autogenous MSCs significantly lower (by 4.45 ± 1.86°) than valgus deformity of the distal segment of their left (control) femur (without transplanted MSCs) ($p = 0.028$).

Likewise, valgus deformity of the distal part of the right femur in the animals of Group B (with the excised bone bridge and transplanted MSCs) was 4 months after the therapeutic transplantation of MSCs significantly lower (by 3.66 ± 0.95°) than valgus deformity of the distal segment of their left (control) femur (without transplanted MSCs) ($p = 0.001$).

However, no statistically significant difference was found in the length (i.e., the lengthwise growth) of the right femoral bone ($p = 0.495$) and its valgus deformity ($p = 0.1597$) between rabbits with transplanted autogenous MSCs (Group A) and rabbits with transplanted allogeneic MSCs (Group B).

MRI examination proved the presence of paramagnetic nano-particles of iron oxide at the site of transplantation of labelled MSCs in the lateral section of the right femoral condyle in 100% of cases 3 weeks after the transplantation as well as 4 months after the transplantation (Fig. 1).

The presence of newly formed hyaline cartilage was confirmed by histological examination at the site of the original defect of the growth plate of the right distal femur in all the animals of both groups A and B (Fig. 2). No histological signs of allogeneic transplant rejection were found in the microscopic sections of the distal physis of the right femur (experimental animals of Group B with implanted allogeneic MSCs). A bone bridge was histologically demonstrated in all cases in the distal physis of the left femur with an iatrogenic growth cartilage defect without transplanted MSCs (Fig. 2). Pearls reaction in the histological preparation of the distal physis of the right femur of all the animals of both groups A and B was positive. The presence of particles of the lipophilic stain DiI was confirmed by immunofluorescence in all the histological samples of the distal growth zones of the right femoral bones with the exception of one case in Group A (MSC autotransplantation) and one case in Group B

(MSC allotransplantation) (Fig. 3). Histochemical analysis – collagen – 2 immunostaining (Fig 4) and PAS reaction was positive in all the histological samples of the distal growth zones of the right femoral bones in Group A and B.

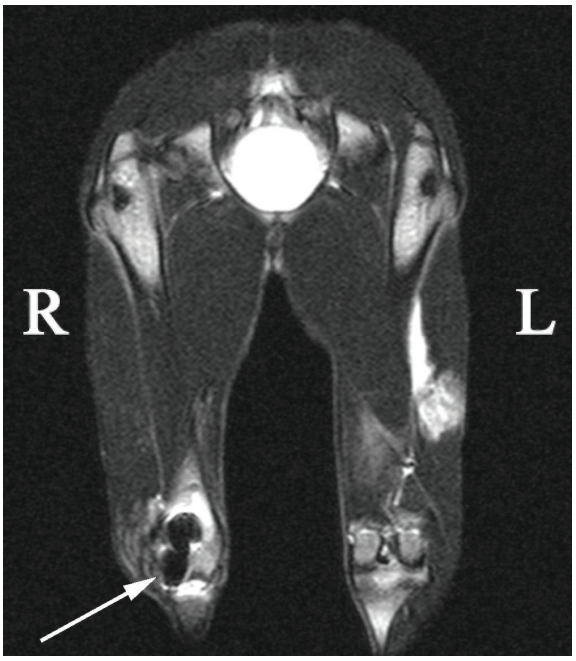

Figure 1. Resovist contrast agent incorporated into transplanted MSCs. The arrow shows an artifact created from paramagnetic iron nano-particles with Resovist contrast agent incorporated into transplanted MSCs (rabbit A5).

Discussion

In our experimental study, we tried to find whether there is difference in the results of a bone bridge treatment by its excision and transplantation of mesenchymal stem cells in dependence on the type of the cellular implant used (autogenous vs. allogeneic MSCs). Thus, the fundamental question is whether we can expect the same clinical results from autogenous and allogeneic transplantation of MSCs.

It is appropriate to investigate both methods for future clinical practice. Allografts may be potentially used in both preventive and therapeutic transplantation into the damaged growth plate. The bone bridge formation may be detected approximately 4 weeks after injury by radiological or CT examination or by magnetic resonance [17,18]. If diagnosing a physeal closure by a bone bridge, it would then be possible to use the cultivated autogenous MSCs collected from the patient when the trauma originated. If the bone bridge, representing a risk of growth disorder of the injured bone (9% of the physeal area) [5,13,14], did not form, it would be possible to keep the unused cells in the tissue bank as a potential MSC allotransplant.

The use of progenitors of chondrogenic differentiation, i.e. stem cells of mesenchymal origin, started to present itself immediately after the first promising experiments with transplantation of autogenous/allogeneic chondrocytes [13,14]. Apart from the bone marrow the source of MSCs may also be the synovial fluid, periosteum, adipose tissue and partly also muscular tissue [8,19]. Their collection should not be burdensome for the potential donor or the patient him/herself. To keep MSC allotransplants successfully in tissue and cell banks and to use them without further delay in case of need in an acute injury of the growth plate if the future shows that it is possible in experiments and subsequently in clinical practice, the method of treating physeal plate traumas by preventive transplantation of MSCs would appear perspective [6]. A suitable scaffold that would provide the cells not only with sufficient space and mechanical support for growth but also with an adequate supply of nutrients could be prepared e.g. on the basis of IPN (Interpenetrating Polymer Network). Rightly timed cascade of other factors that may influence *in vivo* the intercellular matrix is needed apart from the initiation of cell differentiation by supplementing some recombinant factors. In the abovementioned cytology laboratories, the differentiation scheme with the use of TGF-β1 [15] was repeatedly effective in experiments. In general, TGF-β1 and other members of TGF superfamily (BMP2, BMP4) are used for chondrogenic differentiation of MSC cells and TGF is the most „traditional”. Most strikingly BMP2 is also

used in several studies to promote chondrogenesis although it preferentially stimulates osteogenesis. To our knowledge more than growth factor used culture conditions are more imortant for targeted differentiation. As chondrogenesis and osteogenesis display similar initiation processes (nodule formation and collagen deposition) related molecules sucg as TGF and BMP can substitute each other. Our in vitro observations and published articles by other authors indicate that osteogenesis takes place mainly in confluentlike 2D culture while chondrogenesis requires high cell density 3D culture and reduction of cell adhesion to rigid surface. Other supplements in differentiation media (dexamethasone and ascorbic acid) are stimulatory for collagen biosynthesis without any direct effect to differentiation signalling.

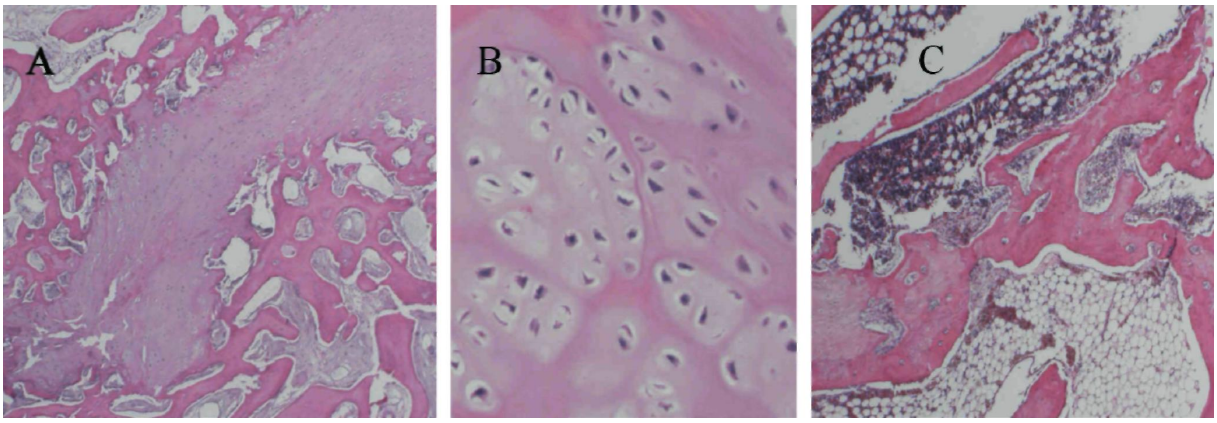

Figure 2. Histological examination of the distal femoral physis in rabbits after autogenous MSC transplantation (HE stain). A – after autogenous MSC transplantation into the femoral defect (magnification X 40 – rabbit A10). B – after allogeneic MSC transplantation into the femoral defect (magnification X 100 – rabbit B3). C – left femur physeal defect without MSC transplantation – bone bridge was formed (magnification X 20 – rabbit B6 – left femur).

The surgical procedure and method of creating the iatrogenic defect in the growth cartilage was chosen in this study on the basis of good experience with methodology used in our previous experiments [6,7,13,14] and in similar studies of other authors [9,20].

These rabbits attain full femoral maturity at approximately 4 – 6 months of age [9,20,21], our observation period (5 week by the operation, 4 months observation period) consists with this reality. The iatrogenic damage of the growth cartilage and in order to make the biggish defect (7 – 9% of growth plate area) described Janarv [5].

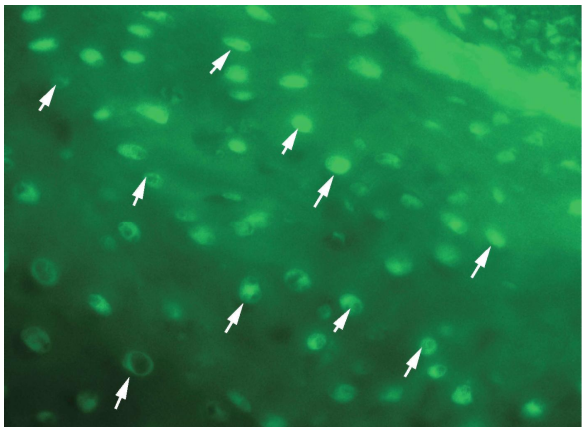

Figure 3. Immunofluorescence stain DiI in chondrocyte membranes (arrows) differentiated from implanted allogeneic MSCs (rabbit A5X 400).

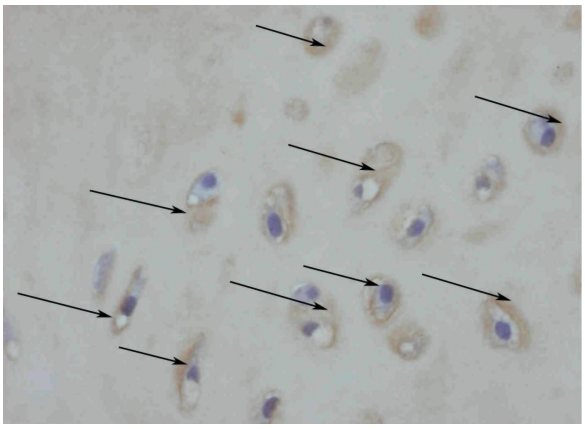

Figure 4. Histochemical analysis – collagen – 2 immunostaining (arrows), positive result (rabbit A2 X 100).

Radiological examination is easily accessible and allows determining with a relatively high accuracy both the length of the x-rayed bone and its possible angular deformity. The method of measuring the femoral bone length in the rabbit was based on Janarv's original study [5], whereas the measuring of the extent of valgus deformity had been consulted with specialists on descriptive geometry previously for the needs of earlier studies of the authors [6,7].

The detection of paramagnetic iron nanoparticles of the phagocyted MSCs in the preparatory phase *in vitro* using MRI is very favourable. In live animals it allows us to detect, i.e. before the end of the experiment, their presence at the site of transplantation, or to find their extinction or travel from the destination site, etc.

Histological examination of microsections stained with haematoxiline and eosin (HE) gives a reliable answer to the character and quality of the cartilage in the iatrogenically created defects. The results of our examinations were fully in accordance with the established hypothesis and also corresponded with the results of several similar studies [9,20]. The presence of bright blue granules in the chondrocyte cytoplasma (Resovist combined with Berlin blue – Pearls reaction) and immunofluorescence stain CM-DiI [9] reliably determined the origin of the cells from the MSC transplant used by out team.

The results of our work indicate that the transplantation of mesenchymal stem cells into the physis at the site of the excised bone bridge may prevent the shortening of the affected bone and the occurrence of angular deformities. At the same time, the study confirmed in an animal model that there is no qualitative difference (hyaline cartilage) in the character of the newly formed cartilaginous tissue in the use of autogenous vs. allogeneic MSCs. In clinical use, both methods of MSC transplantation (autoand allotransplantation) may have their justification, in dependence on the given clinical case and circumstances of treatment.

Conclusion

Allogeneic as well as autogenous transplantations of MSCs into the growth zone defect after the bone bridge excision prevented limitation of the bone growth lengthwise and prevented the development of its angular deformity. Concurrently, no difference was found between the results of allogeneic and autogenous transplantation of MSCs. Both from the viewpoint of the lengthwise bone growth and its potential angular deformity. From the viewpoint of the quality of the newly formed cartilaginous tissue at the site of the original physeal defect. Following autogenous as well as allogeneic transplantations of MSC, the presence of hyaline cartilage was histologically confirmed at the site of the treated growth plate defect. In the case of allotransplants, no histological signs of their rejection were found. At the same time it was demonstrated in vivo and in vitro that the chondrocytes newly formed at the site of the physeal defect originated from the transplanted mesenchymal stem cells.

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CAIcal: A COMBINED SET OF TOOLS TO ASSESS CODON USAGE ADAPTATION

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Background: The Codon Adaptation Index (CAI) was first developed to measure the synonymous codon usage bias for a DNA or RNA sequence. The CAI quantifies the similarity between the synonymous codon usage of a gene and the synonymous codon frequency of a reference set.
Results: We describe here CAIcal, a web-server available at http://genomes.urv.es/CAIcal that includes a complete set of utilities related with the CAI. The server provides useful important features, such as the calculation and graphical representation of the CAI along either an individual sequence or a protein multiple sequence alignment translated to DNA. The automated calculation of CAI and its expected value is also included as one of the CAIcal tools. The software is also free to be downloaded as a standalone application for local use.
Conclusion: The CAIcal server provides a complete set of tools to assess codon usage adaptation and to help in genome annotation.
Reviewers: This article was reviewed by Purificacion Lopez-Garcia, Dan Graur, Rob Knight and Shamil Sunyaev.

Background

Ever since a relatively high number of DNA sequences were publicly available in databases, several statistical analyses addressing DNA composition have been performed. One of the parameters that first interested the scientist was codon usage [1]. It was soon discovered that a considerable heterogeneity in the codon usage exists between genes within species and that the degree of codon bias is positively correlated with gene expression [2,3]. To quantify the degree of bias in the codon usage of genes, several parameters or indices have been worked out. The

Codon Adaptation Index (CAI) developed by Sharp and Li [4], rapidly became one of the most used indices. The CAI is a measure of the synonymous codon usage bias for a DNA or RNA sequence and quantifies codon usage similarities between a gene and a reference set. The index ranges from 0 to 1, being 1 if a gene always uses the most frequently used synonymous codons in the reference set. The CAI has been used for estimation of gene expressivity and for prediction of highly expressed genes [5-9]; for giving an approximate indication of the likely success of heterologous gene expression [7]; for detecting dominating

synonymous codon usage bias in genomes [3]; for acquiring new knowledge about species lifestyle [3,10]; and for studying cases of horizontally transferred genes [11,12].

Results and discussion

The most important contribution that we aim to provide with our server is to tie together several features, previously existing but disseminated throughout the Internet, and some new features related to CAI calculation and analysis, and to implement them into a single and easy-touse web site.

Description of the CAIcal server

The CAIcal web-server, freely available at http:// genomes.urv.es/CAIcal, calculates the CAI for a group of sequences using different reference sets and includes a complete set of tools related with codon usage adaptation, e.g. the representation of the CAI along a sequence or multialignment and the estimation of an expected CAI value (eCAI).

CAI is calculated following the original method proposed by Sharp and Li [4] but using the recent computer implementation proposed by Xia [13]. In the following subsections we describe the inputs of the server and its main features.

Inputs of the server

The inputs for the server depend on the calculation to be performed. The basic inputs for calculating CAI are the query sequences, the reference set and the genetic code used for translation. The query sequences must be DNA or RNA sequences in fasta format. The server first checks whether the query sequences are a DNA or RNA region that codifies a protein. The reference set required to calculate the CAI can be introduced in a variety of formats, including that of the Codon Usage Database [14]. A direct link to this database is provided in the CAIcal interface. This database contains codon usage tables extracted from GenBank and organized by species. Several of the calculations available in CAIcal, such as the CAI calculation and its representation in a sequence, can be used with two reference sets simultaneously. Therefore, it is easier to compare the codon usage of a gene with respect to the codon usage of two different organisms and check whether it is more adapted to one of them. See the tutorial available from the server home page for a complete description of errors and warnings and for more information about input requirements.

Set of tools

A number of programs and servers that calculate CAI for a gene or a group of genes are available elsewhere, such as CodonW, EMBOSS [15], CAIJava [3], CAI Analyser [8], as well as JCAT [16] and the CAI Calculator [5]. All of these tools represent valuable resources.

The server first provides a number of basic calculations that are also available elsewhere:

(i) The absolute and synonymous codon usage of a group of DNA sequences and other useful parameters such as length, total G+C content and G+C content at the three codon positions, and the effective number of codons [17].

(ii) The CAI of a DNA sequence or group of sequences. This index measures the adaptation of the synonymous codon usage of a gene to the synonymous codon usage of up to two reference sets that can be chosen by the user.

The new features incorporated in this server are:

(iii) An expected value of CAI [18] is determined by randomly generating 500 sequences from the G+C content and the amino acid composition of the query sequences. This expected CAI therefore provides a direct threshold value for discerning whether the differences in the CAI value are statistically significant and arise from the codon preferences or whether they are merely artefacts that arise from internal biases in the G+C composition and/or amino acid composition of the query sequences. The ECAI module that calculates the expected CAI values has been previously described [18]. Additionally, one of the tools included in CAIcal is a graphical local user interface that can be downloaded and allows the calculation of the CAI and eCAI of hundreds or thousands of sequences on a whole-genome scale easily [18].

(iv) The weight of each codon, i.e. the frequency of codon use compared to the frequency of use of the optimal codon for that amino acid in the reference set, can be graphically represented along a DNA sequence using a sliding window defined by the user. This result provides an intuitive visualisation of the changes in the CAI throughout the input and identifies discontinuities that might correlate with informational and/or operational features of the DNA sequence. The CAIscan tool of the CAI Analyser package [8] allows a similar analysis, i.e. scanning a sequence calculating the CAI over a selected window.

(v) A graphical representation can be made of the weight of each codon along a multiple protein alignment that has been translated to a DNA alignment using a

unique reference set for all the sequences of the alignment or using a reference set for each sequence. The inputs for this option are a protein multialignment in clustal format, the DNA sequence of each of the sequences of the multialignment with the same identification field between the DNA and protein sequences and one or more codon usage tables to use as reference sets. This result provides a graphical display that enables the protein sequence alignment to be correlated with the informational/compositional content of the DNA sequence that encodes them.

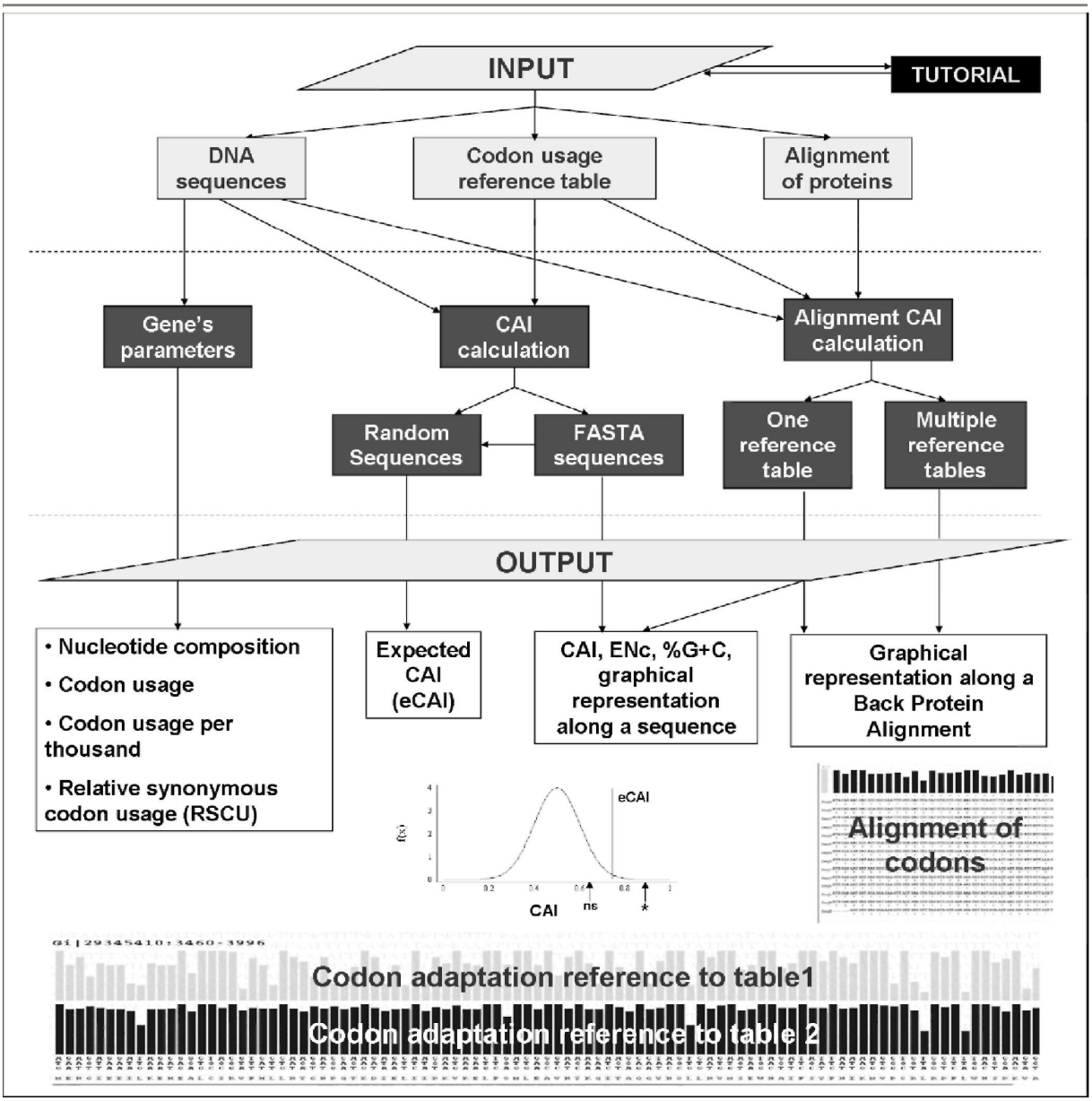

Figure 1. Schematic representation of the options available in the CAIcal server. Using a combination of three inputs (DNA or RNA sequences, a codon usage reference table and/or a protein alignment), the server calculates gene parameters such as %G+C, Relative Synonymous Codon Usage (RSCU) and Effective Number of Codons (ENc), the CAI for one or more DNA or RNA sequences and an expected CAI and represents the CAI along a DNA sequence or in a protein multialignment translated to DNA.

The options available in the server are summarized in Figure 1. All these options are accessible from the main page of the server and several links have been created between them. As an example, after the CAI value of a group of sequences has been calculated, an expected CAI value can be estimated or the graphical representation of the CAI value along each sequence can be visualized. Several parameters used in the calculations, such as the window length in the graphical representation of the CAI along a sequence or the upper confidence limit to estimate an expected CAI, are defined by the user. The results are therefore flexible and fit the needs of the user. For the results, the server produces several tables and graphs together with several text boxes containing the results in a tab-delimited format have been created, which makes it easy to copy and paste them into spreadsheet programs. Finally, a tutorial, a Frequently Asked Questions (FAQ) section and several examples are available from the home page of the server.

Example of how to use the CAIcal server

The CAIcal was used to annotate the genomic discontinuity in the E4 gene of human papilomavirus 1 (HPV1). Papillomaviruses (PVs) are a family of small dsDNA viruses that cause a variety of diseases including cervical cancer. The genome of PVs is modular with three different regions, each of which has a different evolutionary rate [19,20]. These regions are: an upstream regulatory region, an early region that codes for proteins (e.g. E1, E2, E4, E5, E6 and E7) involved in viral transcription, replication, cell proliferation and other steps of the viral life cycle, and a structural region that contains two genes that code for the capsid proteins L1 and L2. A general characteristic of genes encoded in human PVs is their peculiar codon usage preference compared to the preferred codon usage in human genes [21,22], although the exact reason for this poor adaptation to the genome of their host is still unknown. Like other viral genomes, some of the PV genes overlap partially or completely. This is the case of the E4 gene, which is completely nested within the E2 gene in a different reading frame [23]. The function of E4 is not completely understood and its annotation is not very rigorous [14]. The mature E4 protein appears after splicing, with the donor site situated some codons downstream from the start codon of the E1 gene, and the acceptor site situated close to the middle of the E2 gene [24,25]. The fact that most of E4 overlaps with E2, that the mature E1^E4 protein contains a few amino acids from E1 and that the splice sites are not strictly conserved, makes it difficult to determine the true E4 sequence *in silico*. The E4 PVs genes available in the databases are therefore very different in length and similarity. Although the genomes of many PVs have been sequenced, information about the expression of their genes or cDNA sequences is only available for a few of them. One of these is HPV1. In this case, the annotation of the HPV1 E4 gene is confirmed by mRNA data [26]. However, the E4 gene from HPV63, a PV that is phylogenetically related to HPV1 [19,20,27], is longer than the E4 gene from HPV1. The difference is between both sequences is 96 nucleotides located at 5' end of HPV63 E4. We can use the CAIcal server to show that the codon usage of these 96 nucleotides at the beginning of HPV63 E4 is very different from that of the rest of the E4 sequence, measured as the CAI value calculated with the human codon usage as reference (figure 2). This suggests that the acceptor splice site of HPV63 E4 is not well annotated and that the true E4 nested within E2 probably starts downstream from the annotated position.

Conclusion

The CAIcal server provides a complete set of tools to assess codon usage adaptation and helps to annotate genomic discontinuities such as the donor splicing site of the E4 ORF of papilomaviruses.

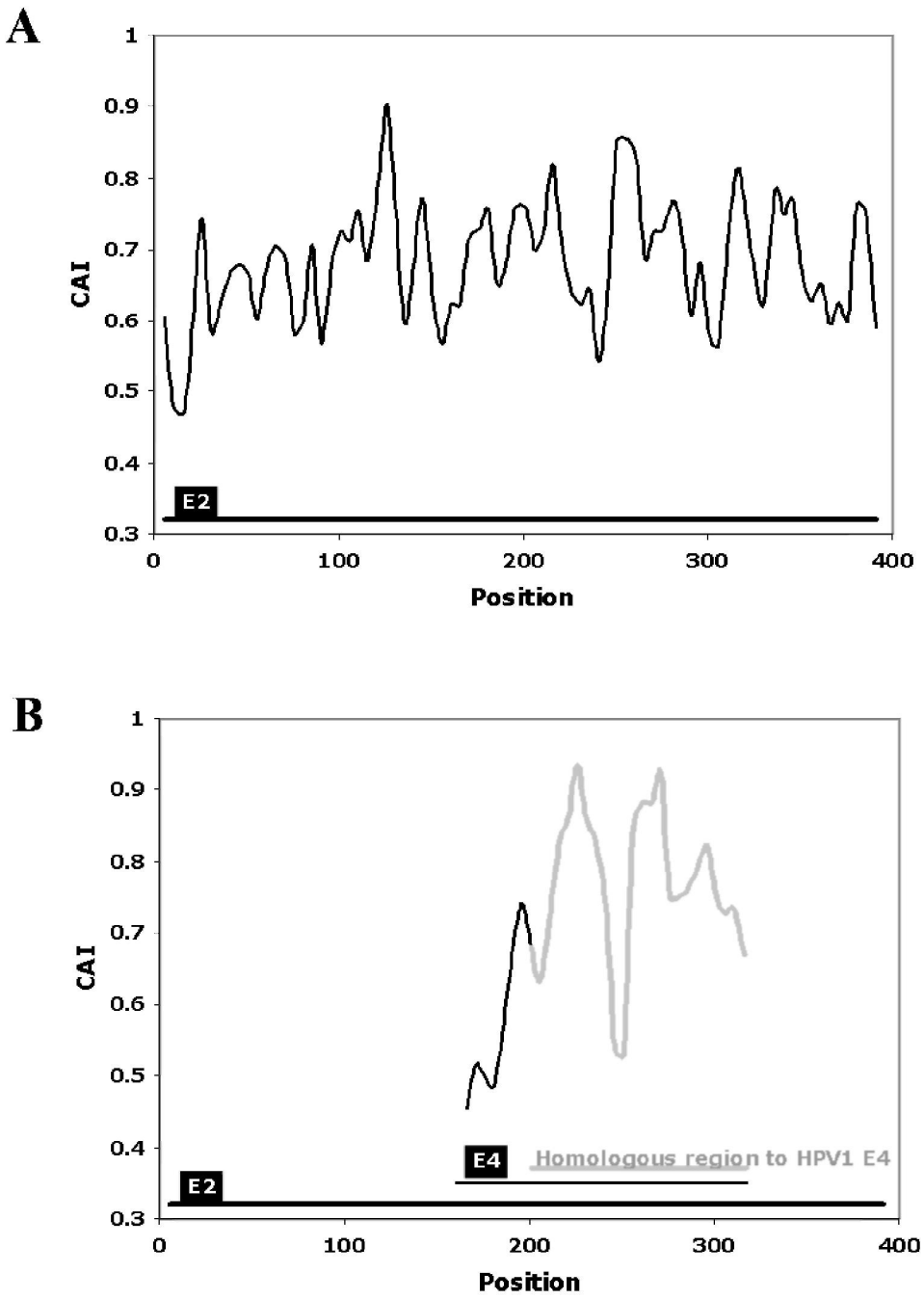

Figure 2. Representation of the CAI, calculated using the human mean codon usage as a reference set, in the DNA sequence that encodes HPV63 E2 and E4. Part A represents the reading frame that encodes E2. Part B represents the same HPV63 genome fragment that encodes E2, but in the reading frame +1, which contains E4. The grey line in B represents the fragment of HPV63 E4 homologous to the closely related HPV1 E4. The black line in B represents the stretch also annotated as HPV63 E4, but which lacks homology with HPV1 E4. Note that the initial E4 region from HPV63, which is not homologous to the HPV1 gene, has an extremely low CAI, which suggests a wrong annotation for the E4 gene in HPV63. This figure was obtained using the output of the calculation of CAI along a sequence of the CAIcal server, with a window length of 11 and a window step of 5.

Reviewers' comments

Reviewer's report 1: Purificacion Lopez-Garcia, CNRS,

Universite Paris-Sud

This article describes a series of tools for the automatic calculation of the codon adaptation index (CAI) and related measurements from input and reference data that have been implemented in a web-based server http://genomes.urv.es/CAIcal. CAI values are useful for a variety of purposes going from genomic annotation and gene expression analyses to the detection of potential horizontal gene transfer events. Although, as pointed out by the authors, a number of freely available facilities providing the calculation of CAI exist already, this new set of tools offers the possibility to obtain some additional estimates. These include the calculation of expected CAIs from randomly generated sequences with the GC content and amino acid composition of the input sequences that can be compared then with the observed CAIs, as well as measurements of the weight of each codon and their graphical representation. An example of the possible utility of these CAI measurements to test and validate annotations is provided. I find that this group of tools accessible online will be useful to the scientific community. I hope that this web-based server will benefit and get improved with the progressive input and suggestions of a wide variety of users.

Reviewer's report 2: Dan Graur, Department of Biology and Biochemistry, University of Houston

A very simple and straightforward tool for dealing with codon usage. I have no other comments.

Reviewer's report 3: Rob Knight, University of Colorado

In this manuscript, Puigbo *et al*. describe their CAIcal web server. CAI, the Codon Adaptation Index, is an important concept relating codon usage to gene expression. Although several software tools online already calculate CAI, CAIcal appears to offer a unique combination of functionality that is not easily duplicated using other tools.

However, the tool in its current form would appear to be a relatively minor advance over existing tools, and I would strongly encourage the authors to consider an extensive overhaul of the software and the manuscript before publication. However, I think the present work contains the seeds of a useful contribution to the field and to the literature, and definitely encourage the authors to persevere, perhaps thinking more carefully about the target audience of the software and the paper.

More attention needs to be paid to the specific contribution of this work if it is to be published as an independent piece of software. No feature of this tool really appears to be unique, e.g. the plots of CAI along a gene and codonby-codon are also in Codon Analyser (as the authors note), many tools allow calculation of CAI against a reference set, etc.

Authors' response: *As we acknowledge in the manuscript, a number of tools are available elsewhere addressing different calculations around CAI. We consider however, that one of the strengths of the CAIcal server is to gather together pre-existing and new features into a single and easy-to-use web site, as you also note in your revision "CAIcal appears to offer a unique combination of functionality that is not easily duplicated using other tools". As an example, after the CAI value of a group of sequences has been calculated, the user can easily (with only a click of the mouse) estimate an expected CAI value for discerning whether the differences in CAI are statistically significant or whether they are merely artifacts. The graphical representation of the CAI value along each sequence can also be easily visualised. In addition, we also want to point out the usability of the server, used to denote here the ease with which people can employ a particular tool. Thus, several of the existing tools that allow calculation of CAI are not web-servers; other require some kind of installation or execution; and some of them provide easy calculations that lack in flexibility. Finally, the server allows to represent the CAI*

value along a protein multialignment back-translated to DNA, a feature currently not available elsewhere.

http://www.biology-direct.com/content/3/1/38

Similarly, the calculations of the expected CAI values are delegated to another tool, E-CAI, that the authors have previously published, but this is not very clear from the description in the paper. If the sole contribution is to tie together several pre-existing features into a single web site, the authors need to make the case much more clearly that this combination will be of use to end users in a way that the individual pre-existing tools are not.

Authors' response: *We have added a new sentence in the paper clarifying this point.*

I think the source code of the standalone version needs a substantial overhaul before publication. It is full of large, error-prone tables of redundant information about genetic codes, for example, which should be dynamically calculated from a compact, standardized and easily verified source (e.g. the NCBI genetic code tables), is essentially without useful comments, mixes presentation and logic, and has many other indicators of poor coding style (for example, it looks as though several separate applications have simply been pasted together).

Authors' response: *Although the main aim of our work was to provide a web-based server for CAI analysis, this was a fair criticism. The source code needed an extensive revision of style and lacked useful comments that could guide the experienced user. We have largely rewritten it and it incorporates now numerous comments about the functionality of each different part. Thus, we have developed the local version 1.3. The source code in the standalone application follows a descendent algorithm rather than several separate applications have simply been pasted together. For the sake of clarity, we have included a file with a detailed description of the CAIcal functions (this file is available from the web site in the FAQs section – http:/ /genomes.urv.es/CAIcal/FAQs.html. The standalone application includes now new functions related with genetic codes to avoid putative error-prone in tables. Though, again, you are right and the coding style could still be improved, the program works well.*

Although I appreciate that the authors have made the effort to produce and distribute a standalone version, the code unfortunately does not inspire confidence in the web site either in this case. Test cases, e.g. using Perl's built-in unit testing framework, would definitely be a useful addition to verify that the calculations are correct.

Authors' response: *This was an interesting suggestion that we have addressed. To verify that the calculations are correct, we show that the results of the two independent programs (the standalone version written in Perl and the web-server written in PHP) are the same. In addition, we have compared our results with the results using other existing programs and the results are not significantly different. A file with some tests we made is available from the web site in the FAQs section http://genomes.urv.es/CAIcal/FAQs.html.*

The utility of the Monte Carlo approach is also somewhat unclear to me, as it appears that the expected CAI could be calculated analytically, along with confidence intervals, using the multinomial distribution. It is possible that this is not feasible for numerical reasons, but some justification of the approach would be useful.

Authors' response: *The expected CAI is calculated analytically from the CAI values of 500 randomly generated sequences with the same G+C content and amino acid composition as the query sequences. However, the Monte Carlo approach is used to generate the random sequences, not to calculate the expected CAI. In this sense, please see also Question 15 at the FAQs section of the server http://genomes.urv.es/CAIcal/FAQs.html.*

I did not find the example especially compelling, but this is a relatively minor

criticism and I understand that it is likely that the authors would want to publish any especially interesting results separately from the description of the tool itself. However, it might be interesting to try to reproduce a well-known conclusion from existing work to show how much easier it is with this workflow than with pre-existing tools. There are many examples in the literature as CAI is such a widely-used technique.

The manuscript and the web site need substantial attention to the quality of the English. I have not corrected minor wording and grammatical errors in this version of the manuscript, but if the authors plan to publish this manuscript regardless of the above comments, I would definitely recommend careful attention to detail, and also removing formatting errors such as the text "Subheading for this section" on page 3. Overall, I think this is a good first attempt and could ultimately be revised into a useful contribution that is more suitable for publication.

Authors' response: *After receiving your comments and the comments of the three additional referees, we have decided to rewrite the code, to revise the manuscript and to publish it. We would like to thank you again for your comments. We think that it is not necessary any further overhaul of the software, as we agree that some changes were necessary in the manuscript and in the source code of the standalone version, and have accordingly been performed. We are glad to acknowledge that the code is easier to read after introducing the comments you suggested. Additional changes in the manuscript include also a second revision of the quality of the English following the recommendations by the NIH Fellows Editorial Board, and some clarifications. We sincerely consider that we have addressed the criticism you raised to the previous version of the manuscript.*

Reviewer's report 3 (second revision): Rob Knight, University of Colorado

The revised versions of the manuscript and software are significantly improved.

Reviewer's report 4: Shamil Sunyaev, Harvard Medical School

This manuscript presents a new online tool to compute codon adaptation index (CAI). Although there are several CAI calculators available online, this new server includes several additional features such as computation of expected CAI and visualization of changes in the CAI along the sequence. The authors also present an analysis of papilomavirus as an example of the server utility. In sum, the manuscript does not report any significant novel scientific findings but presents a tool potentially useful for the research community.

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Materials of Conferences

BLOOD CIRCULATION LEVEL IN GASTRIC WALL IN EXPERIMENT

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The state of gastric wall and vagus nerve blood circulation was investigated rheographically and by the method of hydrogen clearance according to Smith D.R. (1977) in 17 animals (dogs). The main group consisted of 12 dogs, the control one – of 5 dogs. The animals' average age was 3,4 years, their weight – 6,8±0,5 kg. The methodology is based on the polarographic registration of the tissue hydrogen clearance. The registration was carried out through platinous and silver-chloride electrodes introduced into the gastric wall in the course of operation, then – once a day. The main group consisted of experimental animals, which submaximal doses of substrate antihypoxant "reamberin" in combination with prostaglandin were applied to. The given preparations were not used in the control group. The investigation purpose was to define the role of the substrate antihypoxant and prostaglandin in the gastric blood circulation disorders prophylaxis. It was found out that the gastric wall blood flow value (B) in both groups for the moment of the operation performance were high enough and were registered as 185±15,3 and 175±16,3 ml/min /100. Thereat, these values were a bit higher in the main group. In the postoperative period a significant blood flow index decrease was registered in both groups in the space of an hour after suturing. The highest decrease was registered in the control group – up to 59,5 ml/min /100 (2,9-fold), in the main group - 79,8 ml/min/100 (2,3-fold). The analysis of gastric wall blood circulation establishment dynamics in the following seven days testified that the process intensity in the groups differed in the degree of approximation to the norm. In the main animal group the values changed with a greater intensity – on the first day after the operation already B = 126,2±42,1 ml/min /100 (or 46,5% of the original one). Later a relatively uniform blood flow level increase was found out in both groups and with the same value increase. However, these values' increase rate downtrend in both groups was registered since the 4th day. It was found out that a higher blood circulation in the gastric wall in the early postoperative period was one of the conditions for the gastro-intestinal tract motor function recovery. The application of substrate antihypoxant and prostaglandin increasing the level of tissues' oxygenation and microcirculation improves the gastric blood flow indexes both during the operation and in the postoperative period.

The work is submitted to the IV Scientific International Conference "Basic research", Italy (Baia Domenizia), October, 11-18, 2008. Came to the editorial office on 19.08.2008.

BLOOD CIRCULATIONS LEVEL IN VAGUS NERVE AFTER GASTRIC OPERATION

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The state of blood flow in the vagus nerve gastric branches was studied in 17 animals (dogs). The main group consisted of 12 dogs, the control one – of 5 dogs. The animals' age was from 2 to 5 years, they weighing 6,8±0,5 kg. The blood flow was defined in the nerve stem of the nervus Vagus by the hydrogen clearance method according to Smith (1977). Active electrodes were introduced through an epineurium puncture hole subepineurially. The introduction and fixation were performed in the enlargement of a surgery microscope. The blood flow was calculated according to Aukland (1964). The main group consisted of the animals, which submaximal doses of substrate antihypoxant "reamberin" in combination with prostaglandin were applied to. The specified preparations were not used in the control group. The n. Vagus blood flow level comparison in the experimental animals testified that an analogous picture was traced in the gastric wall. In the main group the blood flow values were higher during the operation, than in the control one (66,2 and 46,2 ml/min /100 accordingly). This value fall was significant enough after the operation. Especially in the control group – up to 22±3,1 ml/min /100 (that is 2,1 times less, than the original one). Such a decrease of the blood flow level was registered in the main group as well, but the given parameter value didn't fall lower, than the average mark of 32,1±3,1 ml/min/100. The dynamics of blood flow establishment in the n.Vagus testified that the given process took course most intensively in the nerve tissue on the first day after the operation. Then a relative retardation of the establishment rate was registered. On the average, during a day the value increases by 1,5 ml/min /100. By the end of the investigation the average values of the blood flow volume were at the level of 41,1±6,1 in the main group, and 34,1±6,0 ml/min /100 – in the control one. The findings' analysis testifies that the blood flow values are not constant in the course of the experiment; they are subjected to significant fluctuations. These fluctuations are unidirectional both in the animals with the prophylactic application of the substrate antihypoxant and prostaglandin and without it. The blood flow decreases considerably in nerve stems after the operation irrespective of the original level (twice). The most low blood flow values are registered in the group of the animals, which didn't receive the substrate antihypoxant in combination with prostaglandin.

The work is submitted to the IV Scientific International Conference "Basic research", Italy (Baia

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STRESS-RELATED ADAPTATION CHANGES IN THE SPLEEN DURING EARLY POSTNATAL DEVELOPMENT

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Interconnections between the integrative systems, such as nervous, endocrine, and immune ones, are clearly seen during a stress response. The sources of such a cooperation should be sought at the earliest stages of development. Early negative life events, especially during the neonatal period, resulted in long lasting, irreversible effects on well being. Neonatal stress has implications for host resistance to infection throughout life. Thus, long lasting effects of negative life events on health and disease may be the basis for the individual differences in host susceptibility to infection, malignancy and autoimmune disorders. Age-related aspects of the reduced immunity following stress exposure in terms of possible mechanisms of their development remain not fully understood (**I.G.Akmaev et al., 2002; S.K.Butcher et al., 2005; R.Avitsur et al., 2006).**

The objective of this study was to compare immunomodulatory changes in the spleen as a peripheral organ of immune defense in different age groups of the growing experimental animals under the chronic effect of a severe stressor.

Thirty two Sprague-Dawley rats of the two age groups, each of which included 16 animals, were either exposed to the severe chronic (restraint) stress (**R.Kvetnansky et al., 1970**) with 7 daily 5-hour sessions (eight animals per subgroup) or used as an age-matched control (eight animals per subgroup). The 1st age group contained weaning animals aged 21 days and the 2nd age group included early postweaning animals age 30 days.

After the last session of stress the animals were weighed and euthanized by cervical dislocation. The lymphoid organs (thymus, spleen and mesenterial lymph nodes) were collected, weighed and processed for histological examination. Formalin-fixed paraffin sections were stained with haematoxylin-eosin and immunohistochemically stained by monoclonal antibodies (Serotek, UK) against rat CD8 (T-suppressor/cytotoxic lymphocytes), CD20 (B-lymphocytes) and CD68 (macrophages) surface markers using ABC-method (**J.Polack, 2000)** with subsequent image analysis of the profiles of the immunoreactive cells on the NIKON camera-captured digital pictures using Image Pro Plus 4.5 software.

At the end of the last stress sessions the body mass of the experimental animals was significantly reduced in both age groups ($p<0,05$). Relative splenic mass was also decreased in the experimental animals compared to the age-matched control groups in the weaning and early postweaning pups ($p<0,01$).

The results of the microscopic investigation presented dramatic immunomodulatory changes in different compartments of the spleen which were mainly localized in the splenic white pulp, with red pulp and marginal zones being also involved. The lymphatic follicles of the experimental rats of both age groups were reduced in number and size, lacked germinal centers and were filled with tingable-body macrophages containing numerous apoptotic bodies. Periarterial lymphoid sheaths also decreased in size mainly at the expense of their inner zone. Tingable body macrophages filled with apoptotic bodies were less common for the periarterial lymphoid sheathes compared to the lymphoid nodules. The marginal zone of the lymphoid nodules and periarterial lymphoid sheathes was reduced in width while this reduction was more prominent in the animals of the weaning experimental group.

The immunohistochemical staining for the CD8 of the control animals spleen exhibited immunoreactive cells localized mainly in the periarterial lymphoid sheathes with fewer cells in the red pulp, marginal zone and mantle zone of the lymphoid follicles. The accumulation of the CD8+cells increased with age. After chronic exposure to the severe stressor the number of immunoreactive cells in the periarterial lymphoid sheathes was notably reduced with single immunopositive cells still present in the red pulp and marginal zone. Staining for CD20 revealed concentration of the immunopositive cells in the lymphoid nodules with less dense distribution of the immunoreactive cells in the marginal zone and red pulp. After the last stress session the number of the immunoreactive cells appeared to be reduced in the splenic B-zones of the experimental animals of both age groups. Staining for CD68 demonstrated crowding of the immunopositive cells in the red pulp of the experimental and control animals of both age groups. In the lymphoid nodules and the marginal zones of the control rats they were very rare and in the splenic T-zones they were absent. In the experimental animals single immunopositive cells were also visible in the splenic T-zones.

Quantitative immunohistochemical analysis showed that the volume and numeric density of the CD8+ lymphocytes was significantly reduced in the weaning and early postweaning ($p<0.001$) experimental rats against the control groups of animals. The volume and numeric density of the CD20+ lymphocytes was meaningfully decreased in the weaning ($p<0.001$) and postweaning ($p<0.05$) experimental animals accordingly. The changes in the volume density of the CD68+ cells in the experimental animals did not reach the level of significance in both age groups compared to the control rats.

The results obtained demonstrated the presence of the immunosuppressive changes in the T- and B-zones of the splenic white pulp in the growing body which were most pronounced in the weaning period of the early postnatal development.

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SINGLE INTRATUMOURAL INTERLEUKIN-2 TREATMENT *PRIOR* SURGERY: LOCAL AND SYSTEMIC EFFECTS ON TRANSPLANTABLE MOUSE MAMMARY ADENOCARCINOMAS

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The problem of local and systemic breast cancer recurrence after radical mastectomy in both medical and veterinary practice is not yet solved. Earlier we showed in a number of mouse models of breast cancer that neither mammary carcinoma extirpation alone nor local *peritumoural* (PT) interleukin-2 (IL-2) therapy did not prevent deaths of recipient mice from tumour growth/relapse and/or lung metastases. However, advanced transplanted mouse mammary adenocarcinomas (MACs, therapeutic models) with early appearing tumours were more sensitive to single PT IL-2 therapy than late appearing MACs. It was also shown that single *intratumoral* (IT) IL-2 injection caused systemic anti-cancer effect in DBA/2 mice bearing advanced SL-2 transplanted lymphomas. We hypothesised that IL-2 therapy *prior* surgery may cause both local and systemic effects in preventing of appearance and growth of recurrent MACs and/or metastatic disease. To this end a combined surgical mouse model with a single *intratumoural* IL-2 application one week before MAC extirpation was developed. Growth rate of *contra lateral* untreated and non-extirpated "marker" MAC was used to visualise the IL-2 systemic effects. The aim of this research was to compare local and/or systemic effects of a single IT IL-2 application *prior* surgery in early and late arising transplantable MAC models. Local IL-2 effect was assessed before surgery by right-sided tumour growth delay and after surgery by frequency of appearance and growth rate of recurrent tumours. Systemic IL-2 effect was estimated observing the behaviour of untreated non-extirpated "marker" tumour and metastasis incidences. Efficacy of the IL-2 treatment was evaluated by the improvement in mouse survival in IL-2 treated groups *versus* controls. Surgery was provided under non-inhalation zoletil/vetranquil anaesthesia. All the recipients with advanced MAC loosing body weight were euthanized by cervical dislocation; tumours and organs of possible metastasis were analysed by gross morphology during *post mortem* examination and by histopathology.

Transplantable BALB/c MAC (Bc-MAC) in syngeneic males was used as an early appearing model having short latent period (lag<2weeks). Bc-MAC was previously characterised in female recipients as a fast growing non-metastasising MAC. Here, syngeneic males (n=33) were injected with 10^6 tumour cells (TC) *per* mouse at the right axillary fat pad (AFP) at day 0. At day 6 *post* transplantation (*pt*) all the recipients were additionally injected with 10^6 TC *per* mouse at the left AFP to produce a "marker" tumour. At day 10 *pt* single $1x10^6$ IU dose of IL-2 per mouse was applied IT to the right-sided MAC of an average size of 4.27±0.08mm (n=16). Control males bearing right-sided MAC of average size of 4.26±0.07mm (n=17) received physiologic solution in the same manner. At day 15 *pt* all treated right-sided MAC were surgically extirpated; almost all left sided "marker" tumours were palpable at this time.

Transplantable Wnt-1 induced MAC in C57BL/6 (B6-MAC, transplanted from original spontaneous transgenic MAC) females was used as a late appearing model having long latent period (lag>2w) of tumour growth and metastasis to the lungs in 67% of cases. Syngeneic B6 females (n=27) were injected with 10^6 B6-MAC TC *per* mouse at the right AFP at day 0. At day 9 *pt* all the recipients were additionally injected with 10^6 B6-MAC TC *per* mouse at the left AFP to produce a "marker" tumour. At day 29 *pt* the first subgroup of right-sided MAC (n=16) having latent period less than 4 weeks ($lag^{<4w}$) reached visible size and were selected for the IL-2 therapy; the second subgroup (n=11) still had palpable or none right-sided MAC at this time (long latent period, $lag^{>4w}$). Right-sided $lag^{<4w}$ MAC (6.1±0.8mm, n=8) were treated IT by a single $1x10^6$ IU IL-2 *per* mouse at day 29 *pt*. Control $lag^{<4w}$ MAC (5.3±0.9mm, n=8) received physiologic solution in the same manner. At day 36 *pt* all treated $lag^{<4w}$ right-sided MAC were surgically extirpated; none of the left-sided "marker" tumour was visible. Only at day 42 *pt* $lag^{>4w}$ right-sided MAC reached visible size (5.7±0.4mm, n=7) and were treated IT by a single $1x10^6$ IU IL-2 *per* mouse; control $lag^{>4w}$ right-sided MAC (6.0±0.3mm, n=4) received physiologic solution in the same manner. At day 49 *pt* all $lag^{>4w}$ right-sided MAC were surgically extirpated; none of the left sided "marker" tumour was visible at this time.

All the recipients with advanced MAC loosing tumour and body weight were euthanized by cervical dislocation; tumours and organs of metastatic potential were analysed by gross morphology during *post mortem* examination and by histopathology. Local IL-2 effect was assessed before surgery by right-sided tu-

mour growth rate delay and after surgery by frequency of appearance and growth rate of recurrent tumour. Systemic IL-2 effect was estimated by frequency of appearance and tumour growth rate of untreated non-extirpated "marker" tumour and metastasis incidence. Efficacy of the IL-2 treatment was evaluated by survival improvement in the IL-2 treated groups *versus* survival of the control mice.

Previously we published that single PT $2.5x10^6$ ME IL-2 treatment caused inhibition of early appearing transplantable Bc-MAC in the therapeutic mouse model. However, only trend to Bc-MAC inhibition was observed here after a single IT $1x10^6$ ME IL-2 treatment. Possible explanations were more aggressive tumour growth in male recipients, smaller IL-2 dosage used, and/or another set of application: IT *versus* PT. For further analysis 2 subgroups of individual tumours were distinguished within Bc-MAC model at the time of a single IL-2 application (day 10 *pt*): (1) $lag^{>10d}$ having palpable tumours ≤ 5mm of size, n=23 (12 treated males and 11 controls), and (2) $lag^{<10d}$ having visible tumours >5 mm of size, n=10 (5 treated males and 5 controls). Analysis of tumour growth rate within $lag^{>10d}$ subgroup at day 13*pt* showed significant delay in tumour growth rate of the IL-2 treated MAC (5.78±0.59mm, n=12) *versus* this parameter in controls (7.65±0.62mm, n=11), $p<0.05$. However, single IL-2 injection in the visible at day 10 $lag^{<10d}$ Bc-MAC resulted in a tendency to tumour growth stimulation; probably IL-2 treatment was applied too late to these tumours.

In the late appearing B6-MAC model there was no tumour growth inhibition/stimulation found in the firstly treated $lag^{<4w}$ subgroup (day 29 *pt*). Treated later $lag^{>4w}$ subgroup (day 42 *pt*) showed tendency to tumour growth rate stimulation.

Finally, before surgery we detected significant local tumour growth rate delay in the only $lag^{>10d}$ Bc-MAC subgroup. Importantly, both early appearing $lag^{<10d}$ Bc-MAC and late appearing $lag^{>4w}$ B6-MAC treated MAC exhibited trend to tumour growth stimulation. These results are in accordance with previously published data in mouse models of breast cancer and show that IL-2 therapy effect is strongly dependent on duration of latent tumour growth period, initial tumour size, and time of application. The IL-2 therapy advantage is proposed to be tightly dependent on a step of tumour-host interactions and, therefore, to be beneficial for a recipient only within a distinct phase (hypothesis of "closed frames" success). Consequently, this "closed frames" successful phase should be clearly recognised in an individual recipient before the IL-2 application.

Analysis after surgery showed, that frequency of cases with relapse was 50% in both the IL-2 treated and the control Bc-MAC. However, significant delay in recurrent tumour appearance and re-growth rate in treated mice comparing with these parameters in controls was observed. Early after surgery (before day 36 *pt*) treated mice survived better comparing with survival of controls that died bearing large recurrent right-sided Bc-MAC and pronounced metastases in the lungs. The longest treated survivors *versus* controls also demonstrated improved survival (after day 42 *pt)* due to absence of recurrent Bc-MAC, delayed marker tumour growth, and postponed metastasis spread. Average survival of treated males, however, was not prolonged, probably, due to the higher metastasis rate in the IL-2 treated males died between day 36 and 42 *pt versus* lower metastasis rate in the controls died within the same time. Similarly to all our previous observations, the longest survivors in Bc-MAC model were from the IL-2 treated group.

None recurrent B6-MAC was observed in both treated and control groups of B6 females validating our approach to use *contra lateral* marker tumour to detect the systemic IL-2 effect in this model. Interestingly, incidences of the cases with marker B6-MAC growth were higher in the IL-2 treated females in both $lag^{<4w}$ and $lag^{>4w}$ subgroups. However, the marker growth rate was significantly delayed in the treated *versus* the control females when calculated for the only tumour bearing females. This lead to improved survival in the IL-2 treated $lag^{<4w}$ subgroup (71% versus 50% in control) and the similar survival in the IL-2 treated $lag^{>4w}$ subgroup (57% versus 50% in control), day 105 *pt*.

Finally, clear indications of both local and systemic effects of a single *intratumoral* IL-2 treatment *prior* surgery in both models were registered. Survival was prolonged for distinct proportion of $lag^{>10d}$ recipients in early appearing Bc-MAC model and for $lag^{<4w}$ subgroup in late appearing B6-MAC model. Single IT IL-2 application in a schedule used, however, with precautions may be recommended to treat mammary carcinoma in medical and veterinary practice as both advantages and disadvantages were clearly shown.

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MODIFIED OSTEOSCINTIGRAPHY IN SKELETAL SYSTEM FOCAL LESIONS DIAGNOSTICS

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A traditional method of metastatic lesion foci visualization is the method of osteoscintigraphy with the preparation Technephor-Tc-99m, which represents a sodium pertechnetate lyophilizate and has an ability to get involved in the phosphoric metabolism, be connected with calcium hydroxylate and accumulated in the skeleton.

A standard osteoscintigraphy method supposes an intravenous introduction of Technephor-Tc-99m and a polypositional examination in a gamma-camera after a three-hour interval necessary for the preparation fixation in bones and pathological formations. It should be noted that the preparation is excreted through kidneys and, at their disturbed function, which is especially often detected in the patients with metastases at cancerous lesion of prostate gland, the preparation fixation occurs in the distorted pelvicalyceal system, which can look like a pathological fixation point in lower ribs in many views. In this connection a standard examination was modified by us. The primary administration was exercised directly under the gamma-camera detector resting on the lumbar region to visualize the preparation excretion through kidneys with the first 20 minutes record.

660 metastatic bone lesion suspects were examined. The primary lesion site in 199 patients was diagnosed in the prostate gland, in 279 patients – in mammary gland, in 101 ones – in the womb and ovaries, and 33 – in lungs, in 12 – in the thyroid body (36 patients – other focalizations). In 80% of the cases the patients were followed up after chemotherapy and operative intervention at the primary site. The rest of the patients were sent to be investigated after the initial examination in the Republican Oncologic Dispensary. An X-ray examination was carried out in all the patients beforehand. Thereat, only in 51 patients were supposed to have metastatic processes in the bones. 128 patients were preliminarily examined with the CT.

Due to our investigation: in 573 patients the preparation pathological fixation foci were detected. Most commonly (about 69%) the foci were visualized in the hip bones, back bone (about 60 %), ribs and breast bone (about 443%), limbs (about 21%), cranial bones (about 10%). A single pathologic focus was detected only in 19% of the cases. In the rest situations a multiple affection (from 1 to 12) was detected.

It should be emphasized that in 132 patients (23% of the patients) the detected alterations were wrongly evaluated at the X-ray examinations as degenerative-dystrophic or traumatic ones, or as the alterations of inflammatory character. In 13 patients the osteoscintigraphy method allowed not only verifying the metastatic lesion foci, detected by the CT, but also detecting new, not found out earlier, ones.

The modification of the method with preliminary visualization of kidneys allowed not only detecting the presence of the inadequate filtration-excretion function of kidneys, but also taking the findings into consideration at the following analysis of the focal lesion in the gross per cent of the cases. This data consideration allowed excluding a falsely visualized abnormal fixation focus of the preparation in 51 patients (21 prostate gland cancer patients and 30 patients with a pathological process of other focalizations).

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WOUND SURFACE DEFENCE METHOD

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The infectious complications frequency at operations achieves 10%, the operative wound purulence within the structure of infectious complications having the maximal specific density, achieving 48,7%. The dominant cause of it is the abdominal wall wound intraoperative contamination, consisting in the contact diffusion of ascites bacterial population in there. Most often it occurs during the operations performed concerning acute surgical pathologies attended by the hollow organs' destruction. The main, traditionally used method of the abdominal wall wound walls defence is draping with gauze wads. However, it cannot fully prevent from the wound microbial contamination and its following purulence.

We have developed a postoperative pyoinflammatory complication prevention method based on the operative wound defence during the operation. As the draping material we used the carbon containing bandage (CCB).

It is a sorption band based on the activated carbon cloth developed in the city of Perm. The sorption capacity of the CCB material relative to bacterial cells is at the average17 times higher than that of gauze fabric. The CCB possesses an appreciable quantity of medium and large transport pores, which provide good absorption abilities of the absorbent with regard to medio- and macromolecular toxins and microbial cells; besides, by virtue of its physical and chemical properties, this adsorbent is promising as a matrix immobilizing medicinal preparations for local action on itself. The gauze wads containing the adsorbent are prepared in advance in the form of a wide strip conforming to the wound width and are autoclaved. Hypochlorite in the concentration of 600 mg/l by dipping into the solution for 10-20 min is immobilized on them just before the use. The drape fixation together with the adsorbent is performed by scarce interrupted stitches towards the aponeurosis edges before the infected abdominal cavity opening.

The wound surface defence from purulent effluent offered by us is easy to use, doesn't stretch out the operation time; the adsorbent appears as an effective carrier of an antibacterial preparation - hypochlorite.

The method has been approved in a clinical unit in 215 patients with operations on the abdominal cavity organs; there were no complications in the postoperative period on the part of the operative wound in this patient group registered.

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MORPHOFUNCTIONAL FEATURES OF LONG-TERM ADAPTATION AND INDIVIDUAL DEVELOPMENT IN SPORTSMEN

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Various types of long-term adaptation or individual development are formed at a more or less prolonged effect of some or other adaptogenic factors on the body. In the people with low capacity of inhibitory-relaxation functional system of defence from extreme conditions (IRFSD) irrespective of age the adaptation takes course due to muscle bulk and strength gaining against the skeletal muscles' relaxation low rate, i.e. a **hypertrophic type of individual development** is formed. At the IRFSD medium capacity a **passage type** is formed, and at the IRFSD high capacity a **relaxation type of individual development** is formed. A high relaxation rate and medium muscle strength indexes are indicative of this type (Vysochin Yu.V., 1988; Denisenko Yu.P., Vysochin Yu.V., 2002).

Considerable morphofunctional alterations at long-term adaptation touch not only the neuromuscular, but all the other systems of the body as well. In the hypertrophic type people the hyperexcitability and the CNC inhibitory systems' low activity are registered, the hyperkinetic (uneconomical) blood circulation type (CT) and highly disharmonious constitutional type prevail. The cardiac performance low economical efficiency, a higher level of energy consumption at rest and at testing loads, an increased concentration of energy exchange metabolites, adrenalin and stressor hormones, but a lower level of noradrenalin and anabolic steroids at rest and loads in blood, low stress and anoxia tolerance, a reduced immunological resistance, high incidence of disease and traumatism are indicative for them.

The relaxation type of individual development is the most profitable in all intents. For relaxation type persons the CNC exciting and inhibitory processes' balance, high rate of muscles' relaxation, excellent regulation and movement coordination, perfect reaction to moving objects, that guarantees the sport, everyday and street traumatism minimization, are specific. The most economical – eukinetic circulation type prevails in them, the cardiac performance high economical efficiency, the minimal level of energy consumption, a decreased concentration of energy exchange metabolites in blood, a high rate of reparative processes and resynthesis of energy resources, excellent physical performance and stamina prevail in them. They excel with an increased stress tolerance, twice or trice as seldom they are subject to overwork and diseases, as compared to the hypertrophic type persons. Relaxation type sportsmen, as contrasted with hypertrophic type ones, enjoy considerably greater sport longevity, stand physical and psychological stresses far easier, are subject to various overworks, traumas and diseases 8-10 times as seldom and achieve the highest sport results (Vysochin Yu.V., Lukoyanov V.V., 1987; Denisenko Yu.P., 2007).

With the increase of skeletal muscles' voluntary relaxation rate (VRR) and the formation of relaxation type of long-term adaptation the sport traumatism decreases progressively from 95-100% (at the VRR less than 4,01/sec) to 5-0% (at the VRR more than 9,01/sec) and, therefore, their health improves the same progressively. Our multiyear investigations testified that even in the most traumatic kinds of sport, one can almost fully make away with injuries (except for the traumas emerging at gross violation of game rules by the rival) due to the correct organization of the work-out session aimed at the CNC nervous processes' balance normalization, muscles' VRR increase and long-term relaxation type formation.

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BIOMETRIC METHOD OF DIAGNOSTICS OF DIABETES

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The omnipresent growth of diabetes incidence in economically developed countries and the pathology conditioned by it and causing not only medical, but also social-and-economical problems in the society, defines the topicality of the investigation carried out. However, there are no authentic data concerning the value of individual disposition and pathophysiological mechanisms of its development up to the present moment.

The main task of the carried out research has been the development of biometric methods of II type diabetes predisposition general criteria detection. The offered methods of screening study based on the human dermatoglyphic picture analysis are simple enough, economic; they don't require expensive equipment, chemical agents and highly qualified personnel for their realization; they are noninvasive and easy for the patient. At the examination the dermatoglyphic signs of palms and fingers of both hands are read by means of a special fingerprint scanner, then they are treated with the help of a special computer program. To detect the diagnostic criteria of II type diabetes development we examined 63 II type diabetes patients by the dermatoglyphic method, the control group was made of 63 persons without diabetes, who were chosen by the method of "directed selection" according to their age, sex and nationality. The mathematical classification techniques based on the image discrimination theory were used to treat the material. Due to the research the following dermatoglyphic picture characteristic features have been detected in the II type diabetes patients: the general, total and palmary crest count increase ab, bc, cd, the increase of number and width of palm lines, the axial triradius position in the intermediate t^1 and lateral t^1b position, the palm triradius reduction c, the occurrence of any patterns in the hypothenar and curlicues in the interdigital zone. The developed biometric methods based on the dermatoglyphic picture analysis are able to detect general criteria of predisposition to diabetes development.

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Short Reports

INFLUENCE OF SYSTEMIC FLUOROQUINOLONE ADMINISTRATION ON THE PRESENCE OF *PASTEURELLA MULTOCIDA* IN THE UPPER RESPIRATORY TRACT OF CLINICALLY HEALTHY CALVES

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Abstract

The influence of enrofloxacin administration (5 mg/kg) for five consecutive days on the occurrence of *Pasteurella multocida* in the upper respiratory tract of two healthy calves was monitored over a 10-day period. From nasal swabs of two additional healthy control calves, which received a placebo saline administration, *P. multocida* was isolated throughout the study period. In the enrofloxacin treated calves, *P. multocida* was not demonstrated in the nasopharynx from 48 h after the first injection until two days after the last administration, when *P. multocida* reappeared and proved to be clonal in nature to the original isolates. During the experiment, no change in minimal inhibitory concentration for enrofloxacin of the *P. multocida* isolates was detected (MIC ≤ 0.015 µg/mL). Enrofloxacin concentrations were determined in the plasma by a high-performance liquid chromatography method with fluorescence detection. The PK/PD indices AUC/MIC and C_{max}/MIC ratio were calculated and found to be 1157.7 and 129.8, respectively. Remarkably, the respiratory pathogen *Arcanobacterium pyogenes* became the predominant recovered organism in the nasopharynx of one animal following enrofloxacin therapy throughout the remaining of the experiment.

Findings

In calves, enrofloxacin is frequently used to treat pneumonic pasteurellosis, a disease mostly due to *Pasteurella multocida* [1]. *P. multocida* is a common inhabitant of the upper respiratory tract of calves. To better understand the epidemiology of pneumonic pasteurellosis and the occurrence of antimicrobial resistance, knowledge is needed on how systemic fluoroquinolone administration affects the flora of the nasopharynx in healthy calves. This is important as metaphylaxis is a common practice in the prevention of bovine respiratory diseases. Preventive treatment of "at risk animals" may be associated with a selection pressure leading to antimicrobial resistance or a shift in the population of bacteria present in the nasopharynx.

The aim of the present experiment was to evaluate the influence of consecutive systemic enrofloxacin administrations on the presence and susceptibilities of *P. multocida* strains naturally present in the nasopharynx of clinically healthy calves and to find a relationship with pharmacokinetic/pharmacodynamic surrogate parameters.

Four dairy calves aged 24–28 days were loose grouphoused together during a 5-day pre-experimental period after which the calves were randomly assigned to either a treatment group or a control group. Inclusion criteria were the absence of disease and antimicrobial therapy since birth. The calves were housed two by two in straw-bedded pens (approximately 18 m^2 of floor space per group) in the same automated ventilated stable (17 ± 2°C), but divided by full wooden partitions approximately 1.4 m high. Management and hygienic measures were set up to prevent direct contact between the two study groups. Water and hay were supplied *ad libitum*, and the calves were maintained on an antibiotic-free milk replacer diet twice a day. Clinical observations were carried out and all calves remained healthy during the entire experiment.

The study lasted for 10 days (D0–D9). For five consecutive days (D0–D4), the two calves of the treatment group (weights at D0: 47.0 and 49.2 kg) were injected intramuscularly with 5 mg/kg enrofloxacin (Baytril 2.5%, Bayer, Milan, Italy), while the two calves of the control group (weights at D0: 48.7 and 50.0 kg) received a placebo (5 mL isotonic saline intramuscularly). After disinfection of the nostrils with 90% ethanol, nasal samples were collected using cotton swabs inserted 10–15 cm into the dorsal conchae (Venturi Transsystem, Copan, Italy) every 12 h starting from D0 until D5 and on D6 and D9. Samples were cooled (< 7°C) and further processed within 24 h, starting by vortexing each swab in 3 mL phosphate buffered saline for 10 s. Plasma samples obtained by centrifugation of blood at 4 Ч g for 10 min were taken on D0 at 0, 2, 4, 6, 12, 24 h, on D1 at 48 h, on D2 at 72 h, on D3 at 96 h, and on D4 at 100, 104, 108, 112, 120, 144 and 216 h (the latter intensity to explore the elimination phase) from the treated calves and from the placebo calves at 0 h (D0), and stored at - 20°C

prior to assay. The experimental protocol was approved by the local ethics committee.

In the nasal samples, the numbers of enrofloxacin resistant *P. multocida* isolates and the total numbers of *P. multocida* isolates were determined using a comparative enumerating procedure (duplicate aliquots of 25 µL) on Columbia agar (Oxoid, Hampshire, UK) to which sheep blood (5% vol/vol) and 16 µg/mL bacitracin (1 µg equals 0.0654 U, Sigma Poole, UK) was added with the following concentrations of enrofloxacin (Baytril 2.5%): 0; 0.06; 0.125; 0.25; 0.5, and 1 µg/mL. Reading was performed after 24 h and 48 h of aerobic incubation at 37°C, and consisted of counting colony-forming units (CFU) distributed over each drop zone and averaged for duplicates. The species identification of one *P. multocida* colony per animal per day was confirmed by means of phenotyping and tDNA-PCR and clonality was examined by means of pulsed-field gel electrophoresis (PFGE) [1]. Bacteriological counts were expressed as median and interquartile range, and a non-parametric multivariate analysis of variance (nonparametric MANOVA) for repeated measurements and small sample sizes [2] was performed (SAS version 9.1, Sat Institute Inc., Cary, NC). Evolution of *P. multocida* recovery from the nasal swabs on media without enrofloxacin in both the treated calves and the control calves is given in Figure 1. The non-parametric MANOVA showed that the difference in *P. multocida* isolation was significant between the treatment and the placebo group over time (treatment*time $P = 0.04$, time $P = 0.03$). *P. multocida* was not recovered during the entire experiment on media containing any of the enrofloxacin concentrations. Additional susceptibility testing [1] (range 0.015–1 µg/mL) confirmed no detectable increase in enrofloxacin MIC (≤ 0.015 µg/mL) of seven *P. multocida* isolates recovered from both treated calves on D0 (2) and on D9 (2) and from the control calves on D0 (1) and D9 (2), and PFGE fingerprinting patterns were identical. Whether the clonally identical organisms reappeared in the treated calves either through airborne transmission from the control calves or endogenously via undetected strains, e.g. in the tonsils, is unclear.

To evaluate whether the microbiological effects of fluoroquinolone administration were in line with the current understanding of pharmacokinetic/pharmacodynamic (PK/PD) relationships, two PK/PD parameters were calculated: the maximum plasma concentration/minimal inhibitory concentration (C_{max}/MIC) ratio and the area under the inhibitory curve (AUC/MIC). Theoretically, C_{max}/MIC should exceed 10 and AUC/MIC (AUIC) should exceed 125 to minimize the selection for resistant organisms by bacterial killing also of less susceptible subpopulations (eradication) [3,4].

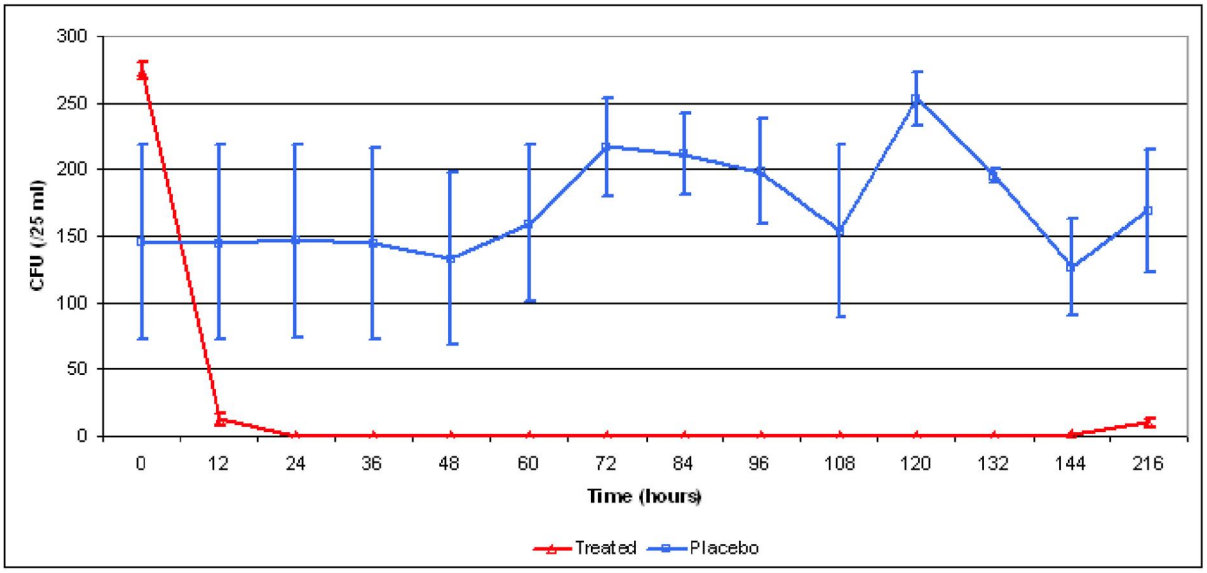

Figure 1. Recovery of *Pasteurella multocida* (colony forming units, CFU) in the nasopharynx of calves treated with enrofloxacin and control calves on media without enrofloxacin. Error bars indicate median and interquartile range.

Plasma concentrations of enrofloxacin and its active metabolite ciprofloxacin were determined using a validated high-performance liquid chromatography method (HPLC) with fluorescence detection. Extraction was performed as described by Manceau *et al.* [5], with minor modifications. Pharmacokinetic analysis was performed using MW/Pharm software (version 3.60, Medi Ware, Utrecht, The Netherlands). The plasma concentrationtime profile could be adequately fitted to a one compartmental model ($r^2 > 0.996$ for enrofloxacin and $r^2 > 0.973$ for ciprofloxacin). All concentrations in the placebo calves were below the

limit of detection (4.6 ng/mL). Maximal plasma concentration (C_{max}), elimination rate constant (k_e) and elimination half-life ($T_{1/2e}$) were derived from the model. The area under the curve from time zero to infinity after the first dose (AUC_0^^) was calculated using the linear trapezoidal method for AUC_0^ and adding the estimated terminal portion of the curve (Ct/k_e), where t is the last time of measurable plasma concentrations after the first dose. Enrofloxacin is deethylated into ciprofloxacin, but the degree of this metabolic process substantially varies within animal species. The mean ratio in AUC_0^^ of ciprofloxacin/enrofloxacin after the first dose found here was 12.3%. This was significantly lower than reported in 8-month-old buffalo calves (27%) and adult cattle (29.9%) [6]. In newborn and one-week-old calves the ciprofloxacin/enrofloxacin ratio can range from 10 to 27%. The ratio is probably lower in young calves due to the lower metabolic capacity at this age [7]. The area

under the concentration-time curve at steady-state over 24 h ($AUC_{0_>2}$4 h) was set equivalent to the AUC_0^^ after the first dose. The ratio of AUC_0^4 h/MIC (AUIC) and plasma C_{max}/MIC was expressed as a dimensionless value. For the isolated *P. multocida* strains the mean AUIC and C_{max}/MIC for enrofloxacin were found to be 1157.7 and 129.8, respectively (MIC for enrofloxacin < 0.015 ng/mL). Even when a conservative MIC of 0.06 ng/mL [1] is taken into account, the thresholds would successfully be exceeded (289.4 for AUIC; 32.4 for C_{max}/MIC). Unfortunately, the obtained values rely on the plasma concentrations and not on concentrations measured in the nasopharynx. Nevertheless, several studies dealing with pharmacokinetics of fluoroquinolones in both plasma and at the site of infection are available in cattle and in line with our observations [8,9]. Recently, it has been shown that fluoroquinolones are a substrate for ATP-dependent efflux transporters which may result in effective drug concentrations in luminal compartments of target tissues [10,11] In addition, during natural courses of bovine respiratory disease, the PK/PD surrogate markers for fluoroquinolones can largely exceed those seen in apparently healthy animals [12].

In one calf of the enrofloxacin treated group, a quasi pure culture of *Arcanobacterium pyogenes* was recovered from D2 (2.9 log10 CFU/mL) onwards and increased in numbers (up to 4.5 log10 CFU/mL at D4) to remain persistent during the remaining time of the experiment. *A. pyogenes* was identified as previously described[13] and the occurrence was observed on the selective media containing ≤ 0.25 µg/ mL enrofloxacin. The latter is in agreement with the study of Yoshimura *et al*. [14] who found a MIC of 0.5 µg/mL for *A. pyogenes* and in accordance with a report by Narayanan *et al*. [15], that support our finding that bovine *A. pyogenes* strains are able to grow on the selective media containing 16 µg/mL bacitracin (equals approximately 1 U/mL). *A. pyogenes* is an opportunistic bovine pathogen associated with chronic manifestations of bovine respiratory disease.

In conclusion, a temporary eradication effect of enrofloxacin for *P. multocida* in the nasopharynx of treated calves was present. This is in line with the current PK/PD approach to prevent the selection of resistance, since the AUIC and the C_{max}/MIC ratio measured in the present study largely exceeded the generally accepted thresholds of 125 and 10, respectively. Although confirmation is needed, our results suggest that other respiratory pathogens like *A. pyogenes*, which are intrinsically less susceptible for enrofloxacin, are able to colonise the upper respiratory tract during fluoroquinolone therapy.

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CHANGE OF PROTEINASE INHIBITORS CONCENTRATION IN BLOOD SERUM IN PATIENTS WITH TYPE 2 DIABETES

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Lysosomal cysteine proteinases participate in many physiological and pathological processes: inflammation, atherosclerosis, immunogenesis, apoptosis [3]. Patients with type 2 diabetes mellitus (DM) are known to have an elevated activity cysteine proteinases [5]. The type 2 DM is also accompanied by rising of the cathepsines B+L and C activity in leucocytes of periphery blood [8]. Rising of activity proteinases is caused either by augmentation of their synthesis or, most probably, by an effect of decreasing of their endogenous inhibitors of the protein nature. Rising of lysosomal cysteine proteinases activity of patients with type 2 diabetes appears to be one of the reasons being responsible for the development of diabetic microvascular complications. Moreover, the increasing activity can reflect the reaction of enzymes to the disturbance of the lipid exchange leading to atherosclerosis being indispensable satellite of type 2 DM. At present it is beyond no doubts that inhibitors play a leading role in regulation of lysosomal cysteine proteinases activity [9]. It is known that the cathepsine B is weakly bound to inhibitors and its activity is easily restored with the decreasing of their concentrations [6], however even the small amount of inhibitors may steadily depress the cathepsine C activity [10].

The aim of the study was to elucidate the serum activity of a_1-proteinase inhibitor (a1-PI) and concentration cystatin C in patients with type 2 DM.

Materials and methods

Under observation there were 26 patients with type 2 DM at the age of 45 to 71. The DM was diagnosed on the basis of the anamnesis of disease, clinical picture and biochemical research according to the criteria of the WHO expert committee on diabetes mellitus. The duration of the disease varied from 1 to 23 years, on the average it has comprised 5.5 years.

Serum cystatin C was measured by ELISA-kit (KRKA, Slovenia). The activity of a_1-proteinase inhibitor was measured by a method based on ability of trypsin to split synthetic substrate of hydrochloride benzoyl-arginine-ethyl ether with formation of benzoyl-arginine [2].

Results and discussion

It is known that the activity serum cathepsin B is very low because of the high contents of proteinases inhibitors [4]. Due to the fact that other proteinases (serine) of blood can participate in degradation of substrate for cathepsin B, they usually mean a serum cathepsin-B-like activity. The research of serum proteolytic activity in patients with type 2 DM (n=14) has shown that the cathepsin B-like activity does not differ from that of healthy people. The α_1-PI refers to α-globulins and has large molecular mass (300-450 кDa). It is considered that it provides 90 % of antitrypsin activity of a serum blood [1]. Cystatin C is a proteiase inhibitor with a low molecular weigth (13 kDa). The serum activity of α_1-PI and serum concentration of cystatin C are shown in the table 1.

Activity of α_1-PI in patients DM was 57 % greater as compared with healthy individuals. The increased antitrypsin activity of blood plasma characterizes intensifying proteolytic processes in serum blood. However, serum cystatin C decreased in 1,8 times that is probably due to consumption of an inhibitor at rising activity of lysosomal enzymes of patients.

Table 1. Contents of serum α_1-proteinase inhibitor and cystatin C in patients with types 2 DM

Inhibitors	α_1-proteinase inhibitor	cystatin C
	IN	nmol/l
Patients, n=21	39,2 ± 8,64 *	38,7 ± 9,51 **
Control group, n=20	25,0 ± 2,64	67,8 ± 2,82
Significance level	p <0,05	p <0,01

Moreover the deficiency of cystatin C is probably the result of the loss of low molecular inhibitor with urine from depression of renal barrier to many proteins (albuminuria), including low-molecular proteins. Previously it was shown the significantly decreased serum - and increased urine apolipoprotein A-I (28 kDa) in patients with chronic glomerulonephritis [7].

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SYNERGETICS ASPECTS: LANGUAGE, CULTURE, MULTILINGUALISM

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The paper raises the question of the three aspects of synergetics: language, culture and multilingualism. Synergetics can throw light upon mechanisms of sense forming. Synergetics treats the text as a non-linear informative medium in which language communication is realized. The mechanism of sense forming is understood not only from the point of view of linguistics but also as an independent reality.

The conditionality of thinking by socio-cultural factors and, in the first place, by the language has been quite thoroughly investigated by philosophers and cultural scholars. The analysis of the connections of consciousness, being and language, has been paid particularly great attention since the so-called linguistic "revolution" in philosophy ushered in by existentialism and phenomenology. It was Heiddeger who initiated treating language as a medium of being and language as the home of being. Gadamer thought language to be not the only instrument of expression, but also a process of a dialogue between being and thinking, between things by themselves and the humanities knowledge of them. Thus language is the whole, the socium through which an individual human being is manifesting himself. Hermeneutics suggests other than instrumentalist approach to the language. In Gadamer's opinion, it would be more correct to consider that a language speaks to us more than we speak it. Languages have existed throughout civilization and the way to implement this is the "speculative game of the language". It is not the subject who is playing, but the game itself is becoming the subject of the playing movement.

A more systematic investigation of the language has been carried out in post-structuralism, which is the philosophy of the language. According to M. Foucault, J. Derrida and J. Delese, the language is a way of interpreting the world preceding any act of reflection. The language is never a language of a speaking person, but the language of conversation things are having with us. M. Foucault conceives the language as a modified nature, as an image of the world. To build the foundation to this thesis, he generalized different national forms of writing: Hebrew, Syrian, Egyptian, Arabic, Turkish, Persian, Mawr and Tatar. All these languages are written from the right to the left following the daily movement of the first heaven. The Greeks, Georgians, Romans and all the Europeans write from the left to the right, following the way and daily movement of the second heaven and the aggregate of the seven planets. The Hindus, Chinese and Japanese write from top to bottom which correlates with the establishment of the nature according to which people have their heads above and feet below. The Mexicans write either from bottom to top, or in a spiral being drawn by the sun during its annual movement through the Zodiac.

On the whole, the philosophic analysis of language is associated in various ways to an analysis of the way of being, which is reflected and fixed in the tongue. It is of interest that the treatment of being differs in different countries and in different ethnoses. "Бытие" (being) in Russian is not equivalent to "Seinschaft" in German. If one renders the word "being" into Ancient Russian it would be "Here I am", which sounds close to "авось" (avos) meaning "may be, somehow, let it be" and nowadays is understood like "we'll get out of it, somehow".

In modern philosophic discourse, new aspects of discussing the intricacies of "language and thinking" have appeared. What deserves the closest consideration is the synergetic approach to the analysis of the language. Synergetics (from the Greek *synergia* = collaboration, co-operation) has been ac-

tively used for more than 30 years in different fields of knowledge, denoting the methodology of investigating the processes of self-organization in complex systems of various origin. Language is one of the most complicated, dynamic and self-organizing systems. This can serve as a basis for extrapolating the laws of nature, which is exposed in the theory of self organization (synergetics) and then applied to the sphere of language and communication.

We are of the opinion that the synergetic analysis of the language makes it possible to acquire a new knowledge of semantics (i.e. sense forming) and organizing communicative processes as well as of the methodology of teaching a language. Let us make a sequential analysis of the above-mentioned directions of synergetic developments in the language.

Sense forming has traditionally been considered as connected exclusively with the activity of thinking man. Consciousness and intellectual activity are the cradles where sense is born. The activity of a human being becomes expedient only through its initial purport. However, this seemingly evident idea was revised by "pre-synergetic" authors, who were dedicated to investigating the problems of the text interpretation.

Classical philosophy looks upon language as a form of thought expression. Non-classical philosophy has come to the conclusion that the meaning of words does not precede the words themselves, but is defined in the context through the development of meaning. Many literary scholars have pointed out that the text organizes the plot itself. A piece of literature is first written and only then, as Borges used to say, if God permits do we find out what we have managed to express. Meaning and senses are being amalgamated in the space of development.

In the framework of a synergetic approach, one can assert that sense is not preset and does not belong to anybody: sense is born continuously in the process of a communicative intercourse. This theory underlies polysemantics and the diversity in interpreting texts.

Sense is not predetermined but appears at the intersection of a number of simultaneously progressing processes as the correlation of separate acts of understanding and as a dynamic phenomenon. The synergetic analysis in general, and in the language in particular, steps forward as a play of evolution in which nothing has been predetermined but the rules of the play itself, whose rules are evolutionary interdictions. The non-linear character of interactions in the language and in the communication process perceived as a dissipative system reveals the process of sense forming. Within a sense of possible space, potent versions are predetermined and formed like events in natural reality, but the senses are actualized and revealed in a dialogue. As it has been pointed out in Hermeneutics, this sense suggests the presence of Another, i.e. another participant of communication.

Let us consider communicativeness, another aspect of the problems of "Synergetics and Language". The communicativeness of synergetics is preconditioned by the fact of its being directed to the cognition of dynamic integrity. The synergetic vision of the world includes recognition of self-activity of being, as well as of the unity of all the ongoing processes including social, thinking and ethical ones. Inside synergetics there is a certain nucleus which is being unseen and in fact is undepictable, nevertheless secures the possibility of synergetic discourse. This is the metaphysic order in the process of making. For conceiving this undisplayed order a new epistemology is required.

While characterizing the parameters of the cognitive situation in synergetics, some authors (V.I. Arshinov, J.P. Svirsky et al.) point out the following peculiarities of it. First of all, in synergetics the dialogue serves as a means of conceptual being and making. The leading cognitive connection in synergetics is the relationship of "I and Another". Synergetic discourse is not directed to expos-

ing laws, but to the dialogue and to creating interpretations.

In the framework of synergetics something deeper and greater than communicative reasoning is admitted. Synergetics suggests an open, communicatively oriented personality. A personal position, as V.I. Arshinov points out, is highly motivated and dynamic. It is "characterized by deviation from binary oppositions: the subjective - objective, the absolute - relative, as well as the artificial - natural and the discovered - created" [1]

The personal position is not equal to subjectivism, it is a highly motivated position of a scholar including the world outlook and moral standards. In the synergetic approach to the process of cognition, the question "What is the object of cognition?" is devoid of sense. There is neither object, nor observer, and the observers position is nowhere. "To know" in the synergetic aspect means "to be able to behave" adequately in situations connected with individual acts or cooperative interactions. [2]

The third aspect of the problem of "synergetics and language" with which we are dealing is the methodological character and is connected with analyzing the synergetic approach to investigating the language. In the system of knowledge, synergetics is referred to the category of interdisciplinary knowledge, that is why synergetics suggests a methodology differing from that of disciplinary organized knowledge.

The process of teaching a language is composed of many components: learning a sum of knowledge (informational aspects); putting senses together (hermaneutic aspect); interpersonal communication (communicative aspect); learning lexical and syntactical rules (cognitive aspect); mastering sociocultural skills of the language communication (moral-ethic aspect) and so on. The question arises if it is possible to organize the process of teaching in such a way that all the aspects of it should interact according to the synergetic principle.

The term "synergetics" suggested by G.Hacken for denoting the theory of self-organization accentuates co-ordinating interaction of parts while organizing the structure as a whole. Synergetic processes are determined by the integrity, configuration of interaction and the place in the structure.

The synergetics concept of self-organization is central not only to the sphere of language and communication (3), but to the description of the world system of languages from separate languages to the linguistic ecosystem level. Revolutionary transformations in the political, economic, scientific and cultural life of the peoples of the world in the 20th century brought about changes in the way the academic community views multilingualism. The negative attitude towards multilingualism that can be traced to the Old Testament's notions of the babel of languages is gradually giving way to the realization of the fact that multilinguality has been a natural human condition throughout the history, rather than abnormality (4). The world system of languages, despite its seeming multilingual chaos and uncontrollable nature of some ten thousand languages, is an open, dynamic, self-organized system which exhibits distinctive synergistic behavior through the combination of the behaviors of individual languages in the network. The vastly complicated network of world languages, according to a detailed study by the Dutch sociologist Abram de Swaan, constitutes an efficient, strongly ordered hierarchical pattern he calls the global language system (5).

The world system of languages maintains its balance through the process of self-organization, relying on the basic ingredients of any synergetic process, notably multiple interactions, without being guided or managed by an outside source.

At present multilingualism has attracted a great deal of attention, and not only in academic circles, in connection with what is perceived as a threat to the plurilinguistic diversity as a result of emergence of the English language as a language of global communication. English, as a global language, is a new phenomenon, without precedent in his-

tory. Thus, the domain of Latin, or any other lingua franca, was limited to the European region, and couldn't be regarded as a language with a global status.

Before a need for a language of global communication arose, the world system of languages was in a stable state, organized on synergistic principles, with different languages taking over some important functions in international communications; thus French was used for diplomatic relations, English was the language of commerce and navigation, German controlled some important branches of science, particularly philosophy, and so on.

A need for a means of global communication brought about some radical changes in the hierarchy of languages. English has spread into all spheres of activities, including world diplomacy, science, cultural and tourist exchanges, information technologies and mass media, and the system of world languages has to readjust itself to new realities so as to maintain its sustainability and prevent a massive loss of linguistic and cultural heritage.

The new stage of synergetic action between the languages of the world is characterized by the recognition of the necessity for a language of global communication, on the one hand, and the need for protecting linguistic diversity. In this respect, Europe's struggles to support multilingualism through incorporating multilingual principles in the EU's legislative acts, is a recognition of one of the characteristics of a self-organized system – its adaptivity to the dangers in the environment without endangering its essential organization. The Vienna Manifesto ("The Cost of Multilingualism"), issued by scholars from all European countries in 2001, put forward some principles and recommendations for the language policies at the new stage of synergetic action between languages. The linguistic future of Europe is envisaged as multilingual, which is seen as a solution to the uncontrollable spread of English and the prospects of English becoming the de facto language of the European Union (6).

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Materials of Conference

FUNCTION OF NATIONAL HERITAGES TRANSFER IN THE KUMANDIN FAMILY: HISTORY AND MODERNITY

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The social and sociocultural situation in conditions of various cultures interpenetration, where a modern family develops and functions, is characterized by many research workers as the identity crisis (G.M. Andreyeva, T.M. Buyakas, A.V. Kuzmin, A.V. Lukyanov, D. Orlov, L.M. Putilova and others). The transformation of traditional family patterns, the shift of emphases of the family group for the purpose of preservation and transference of ethnic experience of life mode unification to the subsequent generations can be one of its manifestations in the sphere of family relationships. The given problem emerges especially acutely before the peoples, the number of which is steadily decreasing. Kumandins belong to such ethnic groups' number.

According to the population count of 2002 there resided 2888 Kumandins in the Russian Federation, among them 1663 persons – in the Altai Territory, 931 – in the Republic of Gorny Altai, 294 – in the territory of Kemerovo Region. Young people under 30 years of age make 1056 (36,6%) persons, among them 461 or 16,0% are still not in their active working age. The productive age Kumandins make 1867 (64,6%) persons. The average age of Kumandins is 40,9 years old. By the present moment Kumandins are integrated into the social and cultural life of the Russian people to a great extent: in most cases the language they use in their every day life and in the family is Russian. A little more than one third of the Kumadin representatives speak their vernacular language (34,9% of the total population of those living in the Altai Territory).

The interest of our research has been the Kumandin family, as the transfer of traditions, customs, and modes of being is exercised, first of all, through the organization of the family way of life, matrimonial and children-parents relationships. In the native philosophy and psychology a significant meaning is given to the investigation of functional features of a family group (V.N. Druzhinin, I.N. Gavrilenko, N.N. Obozov, A.N. Obozova, A.G. Kharchev and others). The following functions are marked out among the principal functions of the family: the reproductive one, the function of primary socialization, educational, communicative, psychotherapeutic and other functions. Within the framework of the given article we are facing the problem of considering how the function of transfer of national family traditions affecting the process of ethnic identification of the younger generation in Kumandin families is realized. The research was carried out in the city of Biysk and in the areas of compact settlement of Kumandins in Krasnogorsk and Solton Districts of the Altai Territory. We specify the data received in the course of inspection of 22 complete Kumandin families residing in the rural area (48% of the total number of the families).

Let us notice, first of all, that traditionally (according to the data of the informers taking part in the depth interview) Kumandin women espoused at the age of 17-18 and men made their families a bit later - at the age of 20. Present day boys and girls marry having reached the same marriage age. According to the Q-data we got the following information: 61% of men-respondents contracted their marriage at 20, 89% of women got married at 18 and later.

The traditions of family making and functioning in Kumandins for the last two hundred years experienced an essential transformation, that was connected with the changes in their socioeconomic status, conditions of life and nature management. The ultimate fact is of prime importance, as the traditional life of Kumandins and their business activity were closely interlaced into the natural processes. According to the results of ethnographic research of XIX-the beginning of XX centuries the blood-tribal relations prevail in the social relations of Kumandins, that contributed to the existence of definite forms and elements of matrimonial-family relationships. So, N.P. Dyrenkova marks out a range of matriarchy features in the matrimonial-family relationships of the Altaic ethnoses, and in Kumandins as well [1, 2]. In particular, it is referred to avenkulat, i.e. especially close relation between the maternal uncle and nephew, - a custom preserving its meaning in Kumandins for a long time; and also the bride price, the ceremony of forgiveness, the duty of son-in-law to help the family of his wife, the ritual of building a hut for the bride, etc. The remembrances of some of these features in Kumandins have lived up to our time.

Thus, during our investigations, the informers A.I. Kazagacheva, V.I. Kukhtuyekova, G.P. Koltychakova, V.T. Belekova, A.P. Teberekova, L.G. Yelbayeva and Ye.I. Tukmacheva, who we are so grateful, told us about the tradition to dress the bride in a hut before the wedding. Before the wedding Kumandins make a spousal hut of nine young birch-trees, which are brought to the forest and heel in there after the wedding ceremony. There is a token that if at least one tree strikes a root, the young marrieds will live a happy family life. On the wedding day the bride coated with a beautiful veil (the face is covered) is shown in the hut, where her girl-friends braid her hair into two plaits. As the informers explain, the two plaits symbolize that from this moment on the woman is no more alone, she has got a match. The bride's

plaits are braided/intertwined “inwards, down”. The married woman doesn’t appear in public uncovered any more. A fire is made before the bride; the meat, which the groom treats the girls braided the bride’s plaits, being cooked in a pot over the fire. As they leave the hut the newly married couple and the girls are poured over with water. In winter they are dipped in snow (“kuryayut”). Then the just married come in the house, where meat cut in solid lumps – bones are not hacked, but are divided in joints – is being cooked in big pots (horse beef usually). The bridegroom puts the slabs of meat into wooden bowls, throws the embroidered towel over his shoulder and the young marrieds go to treat the guests. The round is made with the guests of note, who get the biggest bones with meat. The tribute of respect to the invited guests is manifested in it.

The attention should be paid to one more point of interest of the wedding ceremony organization. In ancient Kumandin traditions only the groom’s relatives are present at the wedding feast. The bride’s relatives don’t take part in the tableful.

To collect the information about the preservation of family actions and traditions we worked out a questionnaire. The analysis of the data obtained testified that 74% of women-respondents do not know about the tradition to dress the bride in a hut before the wedding and, therefore, do not observe it. As for the participation of the groom’s relatives in the wedding celebration, this tradition hasn’t survived at all. 84% of women and 83% of men answered the question “Which of the guests was present at your wedding ceremony?” : the bride’s and groom’s relatives.

The description of the family ritual of forgiveness is of great interest. The groom and bride together with their marriage brokers went after the wedding in the groom’s house to the bride’s parents’ house and asked for forgiveness from the mother and father. The groom’s parents decided when they would go to the bride’s house for forgiveness. Thereat, the forgiveness date was not reported in advance. The young marrieds were no party to the tableful, they aught not to cross the doorstep of the house and waited for their being forgiven near the doorstep on a bench. In the interim the groom’s parents, the marriage brokers paid the bride-price with (money, vodka, livestock) – whatever the bride’s parents claimed for. According to the Q-data 50% of men and 21% of women do not know about the ritual of forgiveness. The action was performed after the wedding by 11% of the rural families taking part in the investigation.

At the same time the changes taken place in the matrimonial-family relations of Kumandins can also be mentioned. Thus, if N.P. Dyrenkova points at the existence of group marriage traces in Kumandins, the studies of the middle of XX century [for example, see 3] and also our researches testify that these traces either vanished completely (except for their reflection in the language) or remained, but in the form of rudiments. In particular, the levirate and sororate (a custom to take in marriage one’s wife’s sisters) ascending to the group marriage are preserved in the rudimentary form in Kumandins. Practically all our informers noted that, if a woman has lost her husband, she will never be left in the cold on the part of his relatives in the Kumandin families. Very often the younger brother takes the child-rearing responsibilities and sometimes husbandly duties towards the widow as well, on himself. However, nobody of the informers mentioned the existence in the man of the right to sexual intercourse with his (native or collateral) elder brothers’ wives, all the more the existence in the woman of the right to sexual relations with younger brothers of her husband, that is described in the research of N.P. Dyrenkova [1, p. 41]. It is the testimony for the fact that in XX century this tradition has completely disappeared from marital relations.

The frames of the given article do not allow describing the transformations taking place in the family ritualism of Kumandins to the full extent. However, the facts described here testify to their significant alternations and in some cases – the total loss of family rituals (a bride stealing tradition, for example). In our opinion, it is connected both with the alternations taken place in the life activity of the whole society and Kumandins as well, and also with a wide spread occurrence of mixed marriages among Kumandins since the beginning of XX century.

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$NiFe_{2-x}Cr_xO_4$ MIXED CRYSTALS' TETRAGONAL PHASE FORMATION PROCESS INVESTIGATION

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In crystals containing transition elements ions, the normal state of which is orbitally degenerated, a spontaneous distortion of polyhedra can occur. Thereat, a crystal field symmetry reduction and transition element atomic energy level removal (Jahn-Teller effect) take place. If a crystal contains Jahn-Teller cations in a sufficient concentration, there appears a cooperative distortion of coordination polyhedra, the crystal symmetry reduction taking place thereat. It is significant that the formation of low symmetrical crystal modifications is attended by a spontaneous appearance of abnormal physical and chemical properties in them. The $NiFe_{2-x}Cr_xO_4$ mixed crystals symmetry reduction is conditioned by Jahn-Teller ions Ni^{2+}. In the given work the results of various history $NiFe_{2-x}Cr_xO_4$ mixed crystals' tetragonal phase formation investigation are quoted.

In the intervals $0<x<1,2$ and $1,4<x<1,9$ the $NiFe_{2-x}Cr_xO_4$ mixed crystals are of spinel structure (the spatial group $Fd3m$) [1]. Over the interval of x from 1,4 to 1,5 a tetragonal phase (the spatial group $I4_1/amd$ with the structure's tetragonality degree $c/a<1$) is formed.

The $NiFe_{2-x}Cr_xO_4$ mixed crystal samples were obtained on the ceramic technology out of corresponding oxides. The thermal treatment was carried out in 10-hour cycles at the temperature of 1100 – 1200 °C. The synthesis fullness was controlled by means of the Roentgen phase analysis. At the computation of the diffusivity coefficient D the formula [2] was used: $h^2 = 2Dv\tau$. Here h – is the formed product layer thickness, cm: D – the diffusivity coefficient, cm^2/c; v – the resultant unit volume expansion at the transferal of one mol of ions; τ – time, sec. When calculating v, the ratio of the bivalent metal oxide density (ρ_{NiO} = 7,45 $г/см^3$ [3]) to the roentgen density value ρ_{roent} of the spinel solid solution on [1] was used. The calculations results are represented in the table. The diffusivity coefficient value D of nickel ferrite (II) at 1200 °C ($D = 1{,}99\cdot10^{-9}$ cm^2/c) is in satisfactory agreement with the one calculated at 1255 °C according to the method of Boltzmann-Matano for $NiFe_2O_4$ $D = 2{,}2\cdot10^{-9}$ $см^2/с$ [4].

The diffusivity coefficient temperature dependence is expressed by the equation analogous to the Arrhenius one [5]: $D = D_0\exp(-Q/(RT))$, with D_0 – is a temperature independent factor, which is formally equal to the diffusivity coefficient at $T\to\infty$; Q – the diffusion process activation energy, kJ/mol; R – a universal gas constant, J/(mol·K); T – temperature, K. The D_0 calculation results are represented in the table. The diffusion activation energy value Q was defined by the graphical method according to the straight line inclination tangent in the coordinates $\lg D - 1/T$.

Table 1. Data for $NiFe_{2-x}Cr_xO_4$ mixed crystal diffusive coefficient calculation

Values of x	Resultant unit volume expansion v	Q, kJ/mol	$D_0\cdot10^3$, cm^2/c
0,0	1,395	173,32	2,789
0,2	1,385	183,81	1,654
0,4	1,375	179,98	0,812
0,6	1,367	183,81	0,670
0,8	1,355	183,81	0,483
1,0	1,352	191,47	0,792
1,2	1,335	218,28	6,364
1,4	1,330	218,28	6,386
1,6	1,347	187,64	0,517
1,8	1,347	183,81	0,668
2,0	1,340	183,81	3,420

In the tetragonal phase existence area an increase of the diffusion activation energy (by 12-13%, see table) takes place. Apparently, in this interval of compositions there appear additional kinetic difficulties for ions' movement. An analogous jump (6-fold approx., see table) is registered in the dependency D_0 (x). For the explanation of the Q (x) and D_0 (x) curves' appearance it was supposed that the spinel formation process kinetics is influenced by the structure factor. Due to the fact that the spinel synthesis was carried out in 10-hour cycles, really the structure formation process ran discretely as well. According to the calculation carried out for the $NiFe_{0,8}Cr_{1,2}O_4$ sample the formed product layer covered about 1,3% of the crystal for 10 hours. At the fall in temperature (cooling down) the structure is distorted due to the cooperative Jahn-Teller effect, a tetragonally shortened spinel being formed. Additional difficulties for the cation dif-

fusion in vacant structure points rise in such a structure owing to the oxygen frame distortion. In the course of the following heating cycles the process of diffusion in "narrow" places is discontinued until the temperature increase takes the lattice distortion off. Because of this phenomenon the spinel structure formation reaction rate decreases as a whole.

For the purpose of the given supposition verification a synthesis of $NiFe_{2-x}Cr_xO_4$ spinels into one stage with an activating additive of potassium chloride at 900 and 1000°C was put into effect. The energy of spinel formation reaction activation at the presence of potassium chloride is less by approximately 50% compared to the energy of a common solid phase reaction. Such a result can be associated with a change in the spinel formation process mechanism: it seems that in the mass transfer process a three-valence metal halide takes part (analogous results were obtained in the work [6]). In the process of $NiFe_{2-x}Cr_xO_4$ spinels synthesis with a potassium chloride additive there is no change in the synthesis duration for low symmetry phases. The obtained result doesn't contradict to the earlier said supposition about the rise of kinetic difficulties in the process with a cyclic character of heating and cooling.

Thus, in the course of $NiFe_{2-x}Cr_xO_4$ mixed crystal structure formation process study it has been established that in the area of tetragonal phase formation with tetragonality parameters $c/a<1$ the diffusion process activation energy value Q has an anomalously high meaning. It can be associated with the kinetic difficulties rising due to the formation of the product with a distorted structure preventing the cation diffusion into the reaction zone at the succeeding thermal treatment cycles. In the course of spinel formation processes in one stage with the potassium chloride participation the formation of low symmetry phases has the rate similar to the one of the cubic phase.

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DEVELOPMENT OF TECHNOLOGY TO PRODUCE NEW SORTS OF CANNED SEAFOOD APPROPRIATE FOR INFANT NUTRITION

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Unlike an adult, an infant is characterized by a rapid growth of the organism and intensive metabolism. Therefore, the efficient nutrition system for infants should provide not only for availability of sufficient food, but also for its specific qualitative composition, appropriate to the gastrointestinal enzymatic potential and metabolic processes changing while the baby shifts to new food, develops physiologically, and grows.

Analysis of Russian baby food market reveals predominance of imports (69%). As to the baby food range, canned fish only makes 3%. The problem of manufacturing the baby supplement foodstuff based on the fish raw materials is not solved yet, because the only national producers of the baby canned seafood such as *Lavr-K*, Ltd., *Faustovo* (Federal Infant Food Factory), *Teledisk Holding Moscow Mill* only meet the demand by 2%.

The fishing industry could be a potential source of raw materials for production of baby foods with a high nutritional and biological value, because fish provides vital proteins, key amino-acids, unsaturated fatty acids, and microelements, while containing few connective tissues and producing a marked lipotrophic effect. In production of the baby canned fish food products, the raw materials, semiproducts, and other materials should meet the requirements set in the current standards and specifications (e.g. SanPiN 2.3.2.1078-01 "Hygienic safety and nutritional value of foodstuff", SanPiN 2.3.2.1940-05 "Children catering", etc.) and be certified by Rospotrebnadzor for production of baby foods.

Supplies are an important factor in development and manufacture of new goods. Russia has developed guidelines on efficient use of raw fish and seafood in production of the baby canned food products which take into account the seasonality and storage life of such raw materials. The most appropriate sorts of raw fish and seafood materials for production of canned fish-and-vegetable food include frozen or chilled fish, as well as frozen skinless fillets of such fish species as cod, seabass, pink salmon, trout, hake, carp, sander, silver carp, Pacific mullet, and sea sander. These fish species have a high content of proteins and essential macro- and micronutrients and are of a high biological value.

Identifying content and balance of macro- and micronutrients in raw materials and finished baby foods, we have used indexes of the nutrient adequacy [1]. The list of such indexes for manufacturing foodstuff for babies under twelve months has been made on a popular belief that the standard food of healthy infants is breastmilk. Therefore, the standard values of nutrient adequacy of foodstuff for babies under twelve months correspond to those characterizing ripe breastmilk. Assessment of the fatty acid balance is made with a criterion which is a specific version of the general criterion of the alimentary adequacy suggested by academicians N.N.Lipatov and A.B.Lisitsyn; this modified criterion describes the whole set and fractions of total mass of saturated, monosaturated, and polyunsaturated fatty acids in the fat stock and finished product [2]. While the child grows he needs more and more new food supplements, therefore, identification of the nutrient adequacy of food products for infants older than twelve months is based on hypothetical quasi-standards of the amino-acid and fatty-acid content of the food for the age group from eighteen months to $2^{1/2}$ years suggested by N.N.Lipatov [3].

Russian specialists have developed a broad variety of recipes of canned mashed and pastelike food complexes for babies: fish-and-vegetables, fish-and-cereals, fish-and-cheese products, etc. The fish component makes 10-16 % of the total weight of such canned food. Other ingredients are: various fine-cut vegetables with low content of vegetable fibre (marrow squash, cauliflower, broccoli, carrot, pumpkin, potatoes, green pea, and spinach), cereals (semolina, oatmeal, pearl barley, peeled barley, rice, maize, and buckwheat); home cheese and flavours. The finished product is ready-to-eat food, i.e. garnished fish, which satisfies the infant's demand for nutrients. The list of manufactured canned fish-and-vegetable products includes *inter alia* "Marrow squash with fish and rice", "Fish with carrots, home cheese, and buckwheat", " Marrow squash with fish and maize", "Fish with vermicelli and carrots", "Fish-and-vegetable stew", "Fish and potatoes", "Fish and broccoli", and "Fish and cauliflower".

Additionally, Russian specialists have developed recipes of baby canned fish without vegetable components. This line is especially interesting as pediatricians often recommend to give a baby monoproduct when he tastes meat or fish for the first time. This allows mothers to identify the baby's reaction to a new food component. Therefore, canned monoproducts are in high demand.

There are recipes of canned fish with the fish content of 45-53 %. The main raw materials are such fish species as hake, sea sander, angelfish, cod, pink salmon, and seabass, while such additives as rice-flour or buckwheat do not exceed 3%.

There are specific requirements to baby foods such as dispersion and thickness, because a baby un-

der twelve months is hardly able to ground food in his mouth, and his digestive system is not morphologically mature and fully functional yet. Knowledge of rheological behaviour of the disperse systems allows us to satisfy these requirements. We have studied rheological properties of canned fish-and-vegetable food and canned fish monoproducts to identify the effect of various factors on their stability. It is found that the optimal thickness characterizes systems with the viscosity values in the range of $\tau_{S.}$, Па 350 – 900, shear stress η^{0}, Па*с 250 – 700, and the particle size of 15 - 75 μm. The specified values are "recommended parameters", which characterize the optimal thickness of the product and are used to monitor the quality under production conditions.

In order to broaden the baby food range, we make researches to develop recipes of fish soups. It is well known that soups are traditionally the main course in our country. The aim of it is to prepare the child's digestive system to protein food. Soups stimulate appetite and activate the gastric juice secretion. Fish and meat soups have a marked effect on the secretion due to the high content of extractive substances (e.g. amino acids, purin bases, etc.). there are special nutrient-technological recommendations on development of soups appropriate to the needs of young babies: the protein fraction should make 3-5 g of total mass, while dry substances in a pureed soup should make 10-15% (in traditional soup with components chopped into small bits – 5-15%), content of amino acids and fatty acids should correspond to the physiological needs of infants. According to standards set by the Federal Research Institute of Nutrition (RAS), pureed soups are introduced when a baby has attained twelve months and traditional soups/ soups with fish balls could be given to a baby from eighteen months onwards.

Our studies allowed us to develop six recipes of soups with optimal content of amino acids and fatty acids for different stages of development of the infant digestive system. The soups have good organoleptic parameters and satisfy the federal recommendations.

Introduction of the developed technologies for production of new sorts of baby canned food products will contribute to provision of babies with foodstuff appropriate to their physiological needs for nutrients and energy.

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UNLOADING GRAIN FROM BUNKER BY SPIRAL-SCREW CONVEYOR

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In bunkers spiral-screwed conveyors are applied to storage of grain at unloading in casings, and also without casings. For reduction of capacity of a drive above a spiral stabilizing plates at various height from it can be installed.

By preliminary researches it is established that grain is unloaded by spiral-screwed working body from that site of the bunker which is most removed from unloading windows. It is necessary to explain the reason of this phenomenon to that the material a screw surface of a spring moves more actively, than the material which is being above the given layer, not having thus of free space for the expiration.

Grain acts in space of a rotating spring and mixs up in an axial direction up to unloading apertures. Speed of movement of layers of grain is not identical and as a result movements of a grain stream the active layer which reason is force of internal friction is formed.

Proceeding from complex internal essence of a bulk material which separate particles are bodies, and all weight has aspiration to current, for the description of behaviour of a "current" loose material it is convenient to assimilate to its some viscous liquid with average, volumetric density and factor of viscosity (internal friction). On the basis of the accepted hydromechanical model dynamics of a loose body can be described the equations similar to equations Navie - Stoks for a viscous liquid.

The received analytical dependences allow to find distribution of speeds at movement of a grain material and to explain features of its unloading from the bunker. Also it is received, that submission of the spiral-screwed conveyor at an unloading of grain from the bunker without a casing in comparison with a casing increases due to an active layer almost twice at the same parameters of a spring and angular speeds of its rotation.

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PROBLEMS OF SECONDARY ENERGY RESOURCES SALVAGING AT PETROCHEMICAL INDUSTRY ENTERPRISES

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For petrochemical industry enterprises the main energy resources use efficiency upgrading direc-

tions for the moment are: a) the energy resources and secondary energy resources use accounting and control system creation; b) the performance analysis and technology updating; c) the exchange of earlier technologies and equipment for advanced ones; d) the energy saving organization on the basis of energy-engineering combination principles.

At the petrochemical industry enterprises the waste energy use is provided, but the degree of their utilization and directions of use are imperfect for the most part imperfect and require a further elaboration.

From the main waste energy use trends adduced in scientific literature only the following ones can be recommended to the industrial isopropyl benzene production: a) the process stream warming-up in the primary and secondary processes; b) the production area and dwelling heating; c) the source and chemically purified water warming-up; d) the ventilation systems' air heating; e) the waste vapor compression heat pump system use within the product and semiproduct dispenser and recovery systems; f) the various parameter cold production; g) the water vapor production.

The exergy method of thermodynamic analysis has a good, constantly developed methodical base and is widely used for the analysis and evaluation of the heat and power systems' thermodynamic perfection.

When developing new energy saving technologies they usually confine themselves to the modernization of separate elements of heat and technology schemes, pay insufficient attention to the creation of integrated systems of heat and refrigeration supply enterprises inclusive of those using low-potential secondary energy resources.

The share of waste energy consumption from the waste treatment facilities is very low nowadays and makes at the average 8-10%. At the high-temperature technology enterprises the share of internal waste sources heat production achieves 50% of the total heat consumption. At the low-temperature processing the utilized heat consumption share makes total only 4-8%.

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CAP STONE BREAKING-OUT PERFORMANCE SAFETY METHODS

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The principle of providing comfortable conditions of work at a mining venture is the development and introduction of techniques and technologies of breaking out useful minerals excluding the occurrence and influence of negative factors of production on the human body. Thereat, it is necessary to take into account that the main reason of traumatism consists in the subjective attitude of the human to hazardous factors of production, and that of morbidity – in the efficiency of preventive measures against harmful production factors and the human's personal physiological features.

At the procurement of natural stone the drilling-and-blasting, drilling-and-wedge and drilling mud methods with unexplosive destroying compositions, cutting with rope and rotary saws, ring cutters, bars and thermal spalling are the most popular ones. The drilling-and-wedge method is applied at the hard rocks procurement, but very seldom – independently. At the procurement of average and low strength stone the cutting method gained its popularity. The drilling-and-wedge method in the specified case is tended to be changed by highly mechanized methods. In the open cast mines, where natural stone is broken off by cutting, it is used in supplementary works, when stabilizing monoliths or giving them a regular form.

A comparative evaluation of all the cap stone breaking-out methods being currently in use has been carried out, inclusive of the method based on plastic substances application. The worksites were certificated on microclimatic, vibration and noise working conditions; the severity and intensity of physical labour mechanized forms being also analyzed.

It follows from the analysis carried out that the work performance using all the considered breaking-out methods, exclusive of thermal spalling, doesn't promote the above-level change of temperature, speed and air humidity of the operating space, provides optimal well-being at the existing effective and effective-equivalent temperatures. The design features of the equipment used at the cap stone breaking-out drilling methods condition optimal and permissive labour provision on the vibration action. By contrast to this, the performance of work using methods based on cutting and thermal spalling is connected with the above-level vibration action, that is partially compensated by the absence of a permanent contact of the worker with the cutting tool and short-run static holdup of the thermal spalling plants during the work. The noise characteristics of the equipment applied at drilling, cutting and thermal spalling exceed the exposure limits. The worst factors are referred to the thermal spalling, that is connected with giving and burning of the hydrocarbon fuel jet under heavy pressure. However, the above-level noise of perforators, rock breakers and explosions at the natural mineral breaking-out drilling methods is mainly low-frequency and broadband unlike high-frequency and narrowband noise of rope and rotary saws, ring cutters and bar machines. This points to the fact that the above-level noise associated with the application of natural stone breaking-out

drilling methods are less dangerous for the human, than those associated with its cutting. The current mechanized forms of physical labour conform to the permissible rates in its severity and intensity everywhere.

It appears from this that irrespective of the carried out preventive measures against the occurrence and influence of harmful production factors, the drilling-and-wedge technologies of stone fracture are the most secure ones on their action on working conditions.

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Materials of Conferences

CYTOCHEMICAL TESTS IN PREDICTING PIGS' PRODUCTIVE TRAITS

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One of the main directions in improving genetic and breeding methods is to search for interior tests to assess precocity and productivity in pigs. The interest arisen to cytochemical methods of determining cell ferments is due to the fact that changes in blood lymphocytes are visualized much earlier than occur morphological and quantitative changes in blood proteins. Cytochemical examinations allow to evaluate conditions of different organs and tissues in a blood drop avoiding biopsy and can be done in dynamics by which there can be determined the state of exchange processes of a whole series of organism systems.

An experiment was made on the experimental training farm of the state breed stud "Tulinskoye" under Novosibirsk State Agrarian University. The object of investigations was made up of the pigs from UKM (universal Kemerovo breed stud pigs). With cytochemical methods applied there was determined the activity of ferments in lymphocytes, that is, succinate dehydrogenase (SD) and glucose 6-phosphate dehydrogenase (G-6-PDG) which are involved in the exchange of energy, proteins, fats and carbohydrates; and the amount of lipids and glycogen - in neutrophils. Blood cytochemical indexes were examined

in the pigs aged 3, 4, 5 and 6 months.

The SD lowest activity in blood lymphocytes was identified in the pigs aged 3 months. The maximal growth of enzymatic activity was found in the pigs aged 4 months (26.13%, $p < 0.05$). The high level of fermentative activity is maintained at the age of 5 months. In the next going age periods the activity of the enzyme was observed to go down. Considerable G-6-PDG activation in blood lymphocytes was revealed in the pigs aged 4 months (39.49%, $p < 0.001$). Regarding the 5- and 6-month age, the enzymatic activity was marked to gradually go down. Investigations in age dynamics of glycogen level in blood neutrophils showed glycogen increase at 4 and 5 months ($p < 0.001$) and subsequent decrease in its concentration at 6 months. Low content of lipids in blood cells was found in the 3-month piglets. Gradual growth of the content of lipids was identified in blood of the animals under 5 months and it was high even at 6 months.

The relationship between cytochemical tests and productive traits of pigs is testified to by correlation coefficients. The negative aspect of the relationship was observed between precocity, SD activity, G-6-PDG, glycogen level and lipids in animal blood cells ($r = -0.214 – 0.393$, $p < 0.05 – 0.001$). As a result of the experiment it was established that cytochemical markers can be applied to assess precocity and productivity of pigs.

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FORECASTING OF PIG PRODUCTIVITY WITH BIOCHEMICAL BLOOD INDEXES

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The use of genetic, biochemical and other interior parameters is an intensively advancing direction. Lipids are the group of substances distinguished by chemical composition and functions. Triglycerides are the most common lipids. Lipoproteins are complex compounds which basic function is to transport lipids. In blood there are several classes of lipoproteins. Low and high density lipoproteins (LDLP and HDLP) are referred to them. Lipoprotein spectrum of blood serum can vary with some diseases in man and animal.

An experiment was made on the experimental training farm of the state breed stud (SBS) "Tulinskoye" under Novosibirsk State Agrarian University. The object of investigations was made up of the pigs from UKM (universal Kemerovo breed stud pigs). The content of (LDLP and HDLP) triglycerides were determined in the blood serum of the pigs. Biochemical indexes of blood were examined in the pigs aged 1.5; 3; 4; 5 and 6 months.

When investigating the content of triglycerides in the blood serum of the UKM pigs, it was established that the maximal rise of their concentration was in the 3-month pigs vs. those aged 1.5 months (28.95%, $p < 0.001$). In the next following age periods the level of triglycerides went down which was further replaced by their building up in the animals of 6 months old (18.42%, $p < 0.05$). The lowest concentration of high density lipoproteins in serum was recorded in the pigs aged 1.5 months. The determination of HDLP amount showed that it was higher in the 3-month piglets. Considerable increase in the HDLP content was identified in the 4-month animals (20.63%, $p < 0.001$) vs. those of 1.5-months old. Gradual decrease of the concentration of the examined parameter was observed in the serum of the gilts in the next following age periods. The UKM animals were marked to have the minimal count of low density lipoproteins at the age of 3 months. The growing level of the determined index was identified in the same-aged at 4 and 5 months, it reached maximal value (2.56 mM/l, $p < 0.001$). The 6-month gilts were found to have the decline of the HDLP content in blood se-

rum. Correlation was revealed between indexes of lipid metabolism and pig productive traits. Correlation dependence between the count of triglycerides, lipoproteins in blood serum and indexes of meat productivity in pigs was of middle and weak degree of relationship. The strongest correlation coefficient ($r = 0.523$, $p < 0.001$) was detected between the serum concentration of triglycerides and lard depth over the 6 – 7th thoracic vertebra. In the experiment there were revealed age changes of triglycerides and lipoproteins in the blood serum of the UKM pigs. The growing level of triglycerides and HDLP in the serum of the 3- and 4-month animals testifies to the more intensive organism energy exchange during this period.

The great concentration of triglycerides in the serum of the 6-month pigs is associated with the period of intensive adipopexis (fat building up).

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Materials of Conferences

THE PSYCHOLOGOLINGUVISTIC ASPECT IN TRAINING TO DIALOGUE SPEECH OF FOREIGN STUDENTS

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Modern achievements of the general psychology, age psychology, and psychology of the person, social psychology, and differential psychology, psychology of abilities and to psychology of training to foreign languages give the right for the methodologists of Russian language as foreign to use data of psychological sciences in practical technique Russian language as foreign more purposefully.

The adult person, including into educational activity, acts in a new role of the pupil. But this new role differs from his role carried out several years ago. The main thing is the changing of their motives of training because knowledge are considered by them as means of achievement of vital plans, the successes, the certain social status. The motivation of cognitive activity of the adult, its activity and purposefulness is defined by the personal importance of knowledge.

Hence, process of training of adult students has:

- to base on previous educational, linguistic, life experience;
- to form at students' knowledge, skills, methods of educational activity;
- to open sense of educational activity of each grade level;
- to develop professional qualities of the person of the future teachers-specialists in Russian philology (speech, pedagogical, etc.).

In our opinion, working with adult pupils it is necessary to consider:

- the level of the previous education received on the native land;
- the culture of the country of the arrived students;
- the communicative needs of the adult pupils caused by a choice of their specialty.

It is necessary to note that the position of the adults in relation to educational activity is a position of the person actively making the decision in conformity with the internal values, motives, belief which have been generated during previous activity - educational or labor (Ananiev B.G., Bozhovich L.I., T.K.Donskaja, I.B.Ignatova, Kuljutkin J.N. etc).

The efficiency of educational activity depends not only on cleanly psychological factors, but also from character of a material so its form represents some language which it's used for transfer to the information [7:186-187].

The transformation of the received information occurs in thinking and is made at a verbally-logic level by means of language, signs and symbols. On the basis of this B.G. Ananiev has allocated three kinds of thinking:

1) subject which is made with the actions expressioning the ideas;

2) evidently-shaped (which is made with search of natural communications on an evident material);

3) verbally-logic which bases on a verbal material [1].

It is necessary also to consider psychological features of perception, fixing and preserving the information. Reflective, identifying and modeling functions of a brain help to check the information in system of an available knowledge.

The training adult foreign students to communicative culture represents a uniform process of formation of system of knowledge, communicative skills and skills which are formed and improved during training dialogue speech (the ability to solve language problems by communicative means in concrete forms and situations of dialogues.

Dialogue is educational unit in which language is presented in a functional-system. Language is a special kind of the maximum intellectual, speech activities of the person.

Dialogue is created as a certain message about subjects and about the phenomena of the validity, reflecting attitude to them and the author's influence on listening person (Dridze T.M., Zimnjaja I.A., Leontiev A.A., etc.).

Dialogue is the complex unit uniting all levels of language and speech (linguistic, semantic, psychological), helps to solve the problems of formation of foreign students' communicative culture.

Training dynamism, stability, plasticity, purposefulness, openness, consciousness, efficiency of dialogue speech defines a level forming communicative culture of foreign students as these qualities determine adequacy of speech behavior to a communicative problem which is solved by authors of created dialogue-statement as a product of speech activity.

So, in such process dialogue - a complex method of training which realizes hierarchy of the purposes of the personally-focused training:

- at a didactic level it provides mastering by various methods, ways of the decision of problems, their generalization and ordering, integration of knowledge and skills;
- at a developing level dialogue provides development of divergent thinking, development of cognitive, research skills, provides true representation about the mathematician;
- at a personal level dialogue awakes an idea, provides an opportunity, to go through unexpectedness of decisions, their originality, amazing, a shock and delight.

Increase of a learning efficiency dialogue speeches, creation of conditions for strong mastering knowledge in a broad sense this word (as knowledge and practical skills) sees not only in perfection of the maintenance of knowledge, but also in perfection of receptions of management by formation theoretical and practical готовностей on the basis of activization речемыслительной activity of students. In other words, it is necessary to arm the student with skills to use rational ways of purchase of knowledge and their processing. It substantially will relieve trainees of an excessive expense of time and energy that as a whole will promote an intensification of process of training to dialogical interaction of foreign students.

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FORMING PERCEPTION OF PIECE OF ART BY JUNIOR PUPILS AT FINE ART CLASSES

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The article deals with the identification of perception development of pupil at the primary school. The author typifies the methodics of forming perception of pieces of art by the pupils at primary school.

Psychologists regard artistic perception of a child as the result of his becoming a person. It is untutored. Not from the very beginning a child perceives a piece of art in capacity of such. In the first instance of the child's development the following is typical: efficient, practical attitude to it (The children palm and touch an image of the picture, stroke it, etc.). In the opinion of some west psychologists, perception of beauty is innate, common to man biologically. Psychoanalysts colligate an artistic perception with a sexual instinct [1].

One can't regard a picture perception of the children without its meaning content. L.S. Vygotskiy specified by an experimental approach, that the perception stages revealed by V. Stern, characterize not the development of picture perception, but the balance between perception and speech at the certain stages of development. A composition of piece of art and the degree of coincidence of meaning and structural picture's center are very important for an artistic perception and understanding of an idea.

The perception of the piece of art is a complex mental process. It supposes a capability to inquire and understand a thing shown, but it is only a cognitive act. An important condition of artistic perception is an emotional side of the perceived and expression of the attitude to it (B.M. Teplov, P.M. Jacobson, A.V. Zaporozhets, etc.). A.V. Zaporozhets mentioned the following: "… an aesthetic perception doesn't resolve into the passive affirmation of the familiar sides of reality, even though very important and essential. It demands that an apprehensive person somehow comes in the imaginary circumstances and takes a part in the actions in the mind's eye" [2].

When perceiving any piece of art it is important not only to have general attitude to the whole piece of art, but the character of relationship and child's value of the separated heroes.

During the development of an artistic perception of school children there is an understanding of the expressive means of piece of art, that leads to more adequate, full, deeper perception of it.

A sure-handed application of music and artistic word influence positively understanding of the picture by the junior pupils and help perceiving the word pictures embodied in them deeper. The interesting techniques develop emotional tenderness and observance awakes a taste.

It is important to form the children's correct assessment of the heroes of piece of art. A conversation, especially by using the problematic questions can help very much. They lead a child to understanding the hidden before second true identity of the characters, motives of their behavior and independent overvalue of them (in case of initial inadequate value).

Perception of pieces of art by the school child will be deeper, if he learns to see elementary expressive means, used by the author to characterize shown reality (color, color combinations, shape, composition, etc.)

Development of the aesthetic perception happens in all kinds of the artistic and household activity of a child. Under the condition of an expert guidance of the elders it can gain a relatively higher level even at the age of school child. Due to the lessons learned the children at the age of 7 – 10 years learn the things and the whole pictures easily. The pupils steadily conceive 'categorically' even unknown mechanisms,

plants, and signs, i.e. as representatives of some group: “It is a car”, “It is a bush”. A syncretistic of the school children is expressed more faint due to the intensifying attention to the relation of the components in the whole and aspiration to find the semantic connections when perceiving an object.

However it is easy to see in school child too some singularity of perception. It is brought on the mistakes in the cognition of the space in a great measure, though accuracy of distinction of the geometrical shapes and correct naming of them by the children over 7 years mount visibly in comparison to the preschool children (L.A. Shvarts, S.V. Muhin, M.N. Volokintina).The first grade pupils have a tendency of turning unknown shapes into the objects (O.I. Galkina, S.N. Shabalin). That is why the junior pupils name a cylinder as a glass, cone (inverted) is a top or roof, quadrangular prism is a column, etc. This means that there are some present troubles in the abstracting shape from the object.

The reason of the stability of many mistakes in perception and shapes’ differentiation is their constant situation of perception. Many of them recognize the straight line, if it takes a horizontal position, but if it is drawn vertically or obliquely, they don’t. The same happens with perception of triangular. If this word children associate only with triangular and only in its one position in the space (Let us say, the hypothesis is located in right and peak is over), all the other kinds of the same shape and even the same quadrangular triangular, place with down peak, isn’t associated with this group of geometrical shapes. Such limitations speak about the extant opacity of the pupils and indivisibility of their perception.

It is very important for graphical perception to compare 2 of the similar, but in some way different things. Such comparison allows emphasizing those distinctive features of the objects, which are typical for them (L.I. Rumyanntseva). The assimilation of dimension is very important for the development of graphical seeing when analyzing any piece of art. An acquaintance with meters and centimeters materializes the graphical features of the objects, and metrical work at the classes of maths, labor, nature study and physical training develop visual estimation, measuring the distance and size. A child learns separating graphical characteristics of the objects and their coexistence at the lessons of fine arts.

Giving peculiarities of the graphical peculiarities and connections, perfection of observation and understanding influence precisely the development of perceiving a narrative (including artistic) picture of school children.

In the result of special training the children begin perceiving not only the picture’s narration, but the compositional peculiarities and many expressive components. The pupils feel a sultry hot day at the harvest time or moist an foggy air on the lake. Such perception is a complex process of a constant development of an idea from the perception of the whole picture (synthesis) to its analysis, then again to the whole picture and to separating less and not noticed before details, which allow understanding the picture’s idea deeper. Selection of the name as the highest form of generalization is quite intelligible to the children at the age of 7-11 years and efficient way of teaching midchildren to parceling-out of the main at the picture.

In midchildhood a special kind of perception, i.e. listening, develops essentially. In the preschool childhood a child oriented to the instructions, requirement, and value of an elder on the basis of perception of his speech. With great relish he listened to the educator’s tale fairy tale. For a pupil listening becomes not only the mean, but a kind of his educational work. Besides, the children listen to the answers, decisions, explanations of their comrades with a critical tendency. Listening and reading become an original form of a brainwork of the children. Such brainwork demands not only division of the some words and understanding of the meaning each of them. Listening to the tale demands the connections between words in the sentence and between sentences, paragraphs, and, finally, units and chapters. As well as in the picture’s perception, the generalization of the whole content is given in the title of the tale and subtitles, given to each of its component, that guarantee deeper understanding of the whole text by the children.

The time perception is very important for development of the school child. In the end of the preschool age, there is a capability to take the position outside of imaginary, the position of spectator (N.D. Nikolenko,etc.)

In the process of developing artistic perception the value of the perceived arises. Special incomparable and the best educational force of the art is firstly in the fact that it gives the opportunity to come into the life, live through the period of life which is reflected in the light of certain ideology, said the greatest Soviet psychologists B.M. Teplov. And the most important is the following: in the process of this experience there are certain relations and moral appraisal, having incomparably big compulsory force, than the appraisals simply reported or teachable [3].

At first the appraisals, occurring in the result of the intrinsic activity of a man, are expressed in the preference of such thing that is liked. They progress together with an artistic perception and take form of the fine estimation of art from the point of view of aesthetic ideal.

Perception of the school child requires not only the readiness of analyzers, but also some experience: knowing things and perception skills. That is why perception forms during the period pf child’s development. The perception perfection can\t be separated from the general mental stature of a child.

Perception pf a school child of any object and its image are the reflection of the whole in the interrelation of its components. These relations of the whole

and its components are changeable and flexible. Any process of perceiving the object as the whole requires the division of its peculiarities, sides, parts (analysis) and connections between them (synthesis). The cognitive work is demonstrated more distinctly in the perception of the complex meaning of the picture, perception of which requires understanding, i.e. it is a form of a complex cognitive work.

The process of developing passes 3 stages: listening, describing and explaining. These 3 stages testify the different degrees of understanding of a given content by a child. They depend on the following:

1) the picture's structure;
2) the degree of narration closeness;
3) the kind of the asked question;
4) the general culture of a child and observational skills;
5) the development of his speech.

That is why a child can show simultaneously different levels of the picture's perception. In other words, the levels can coexist.

The pupils can connect theoretic knowledge with practical activity. The children open up at random and sequent observe the surroundings, connect mentioned facts with data in life, received from the books and teacher's explanations. Theoretical reasoning of the studied new material induces a pupil to check in practice again something discovered by him. The school children acquire tenable and reasonable knowledge. The perceptive culture is a perfection of the whole child's cognitive activity [4].

The perceptive development is a transition form, a conjoint, syncretical, fragmentary perception of the object by the child to the divided and categorical reflection of things, events in their extensional, temporary, casual relations. When developing the perception its structure changes and mechanisms. The children's eye follows the hand movements. A word is a mean of analysis and generalization of perceivable content.

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ON INCREASE IN QUALITY OF EDUCATION THROUGH APPLICATION OF ACTIVE FORMS OF TEACHING

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In order to get a profession a student needs to master a certain amount of knowledge which comprises knowledge of separate disciplines. A generally accepted competence building approach implies that a specialist should not only have knowledge and skills, but also be able to apply the acquired knowledge in practice, he or she should learn to find and use effectively the necessary information, be ready to solve tasks arising from the course of work.

With such approach to teaching students being implemented, it is necessary to intensify active forms of teaching. Introduction of active forms of teaching makes it necessary to change the prevalence of lectures and seminars and to increase the amount of students' independent work under professors' supervision. But the problem does not lies in the necessity to change the correlation of class hours and independent work in the academic plan, but in preparation of the necessary methodological provision that enables students to master certain parts of academic disciplines while doing individual tasks.

In recent years interactive and dialogue methods of giving lectures, solving cases and joint project have spread widely. One of the ways to intensification of students' independent work is self-tests that checks academic material mastering.

In the Finance Academy under the Government of the Russian Federation there was established the Centre for testing within the frameworks of increase in quality of specialists training. One of its functions is to create a multipurpose system of knowledge testing and organization of students' independent work. The Centre coordinates the work of the Academy's departments in the development and accumulation of tests banks and carrying out different types of tests.

Effective use of testing in organization of students' independent work means that there is a bank of tests for independent work on the taught disciplines. While planning classes, it is necessary to include both students' self-training and self-testing on different parts and the whole discipline. Students should have an access to computers to carry out tests (or a network in distant learning).

In the Finance Academy most tests are created in ASR (Adaptive system of testing). Moreover, a multifunctional testing system created at the Faculty of Open Education is widely applied. It provides entrance, training and final control of various parts of disciplines through the Internet-training server. There

are some other approaches to implementing tests in teaching process.

Testing technologies if used competently provides a good tool for measuring the level of knowledge from different viewpoints. Taking the changes in the system of higher education into account it is unadvisable to defy the advantages of testing technologies for intensification of teaching process. It goes without saying that the creation of high-quality banks of tests will demand considerable expenditures but their implementation will enable to diversify and increase the effectiveness of students' independent work.

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ABOUT PROSPECT OF DEVELOPMENT OF THE HIGH SCHOOL SCIENCE ON THE BASIS OF ESTABLISHMENTS OF PRACTICAL PUBLIC HEALTH SERVICES

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Federal Law of Russian Federation « About a science and the state scientific and technical policy »N 127-FZ from August, 23rd, 1996 (last addition from 01.12.2007 N 308-FZ) provides two kinds of scientific activity: research and scientific and technical. In high schools employment by scientific activity, and in treatment-and-prophylactic establishments which carry to sphere of services to the population is necessary, scientific work is not obligatory. Nevertheless, among the practical doctors, the much creatively presented people, capable to invent new medical production and to conduct scientific researches. So, in city clinical hospital №3 of M.A.Podgorbunskogo inventions on neurosurgery, traumatology, anesthesiology, resuscitation, abdominal surgeries, urology, endoskopy are created, radiology and to other specialities and 70 patents for inventions are received. At the international exhibition «Week of high technologies», passing in St.-Petersburg in June, 2003, the hospital is awarded by a silver medal. Doctors act with messages on concrete inventions and results of scientific researches at various congresses, the congresses and conferences. Practical doctors only our hospital protect, as a rule, in the Kemerovo state medical academy more than 40 candidate and two theses for a doctor's degree.

Hospital experience has shown, when clinical chairs of medical high schools work in creative cooperation with highly skilled experts of clinical hospitals, use of a mental potential of establishments of practical public health services can bring the considerable contribution to development of a high school science and to scientific and technical progress to medicine. Results of research work become high school production, and scientific and technical activity – intellectual property патентообладателя. Various application of patented production, including at the professional organisation of patentno-licence trade on internal and the world market, at ability and diligence, can give additional incomes and open new economic prospects to owners of patents.

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ROLE OF STUDENTS' RESEARCH WORK IN THE AREA OF ECONOMIC PROCESSES STUDY WHILE TRAINING QUALIFIED EXPERTS

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At the present stage the students of higher professional economic tertiary education get background knowledge in various areas of economic sciences, nevertheless, the disruption between the theoretical knowledge acquired in the process of training and the demands of modern Russian economics being now in need for new researches and developments is, perhaps, much greater, than before.

There are many samples about it. So, in the course of microeconomics of any economist having experience of work at a real enterprise, the use of one-commodity model bewilders. But in the real market only multi-commodity firms act practically. Any small enterprise performs different kinds of work and services. And any tiniest booth sells tens types of merchandise. But there can be more than a hundred of such commodities at big enterprises. And the main problem facing the administration consists usually in the choice of the right assortment. The full time students having no practical experience of work and studying the course of microeconomics in a HEI simply don't know what managers really concern themselves in the firm. Problems rise to the surface much later, after graduating from the HEI. Having come to the working place the former student finds out that, in fact, Economics is hardly helpful to anybody in the form it was taught.

Several more examples are given here. On the pages of a standard theory textbook there never appears such a concept as "quality", important for providing the firm's working life. Doesn't it deserve a theoretical treatment? Things are not better with the project thinking. The theory presentment is composed

as if the firm turns out one and the same products continuously and unlimitedly long. The expert is, first of all, asked for the solutions concerning business-projects, i.e. single, time-limited tasks a distinct beginning and end and almost always being in need for specification in the course of realization. And the presentment of the theoretical material on price formation is composed as if the firm knows its production demand curve in and out. Besides, in some textbooks of the mean level everything is limited by this very curve, that is still possible to be taken for a simple model of the process. But in the "advanced" editions the student is famously taught to operate with the second differential coefficients, leaving not even a hint to how the analytic expression of the corresponding functions was got. The learner is made solve the problems uneasy even in their mathematical form with the help of these methods. It is obviously supposed that it will help him in the practical management of the firm. But in fact the demand curve is unknown to the manager. More over, it is so changeable, that its any mathematical expression can be considered only as its evaluation with a certain share of probability.

The poignancy of the briefly outlined problem of the theory and practice disruption in the educational process sharply grows by the virtue of the international commitments accepted by our country. Within the framework of the Bologna Agreement already signed by Russia there placed a greater importance on the economic theory as a whole, than within present-day Russian educational programs, while training bachelor's degree holders on economic directions.

The Economics study deepening can consist only in the transfer to the theory teaching focused on practice. This is an uneasy problem and there are difficulties here.

As it is known, new theories in the economic science have usually a high degree of the apparatus formalization, that gives rise to revolutions from time to time. The result of this is the refusal of a part of scientists to go on rounding out the apparatus details and the effort to revise the fundamental principles instead of it. The end of one of such revolutions – the neoinstitutionalistic outbreak of the neoclassics theoretic paradigm – we are outliving now. To change the epoch of "Storm and Stress" there comes a new era of absolutely necessary perfection of the apparatus – the neoinstitutionalism grows together with neoclassic. For the science having such a complex subject to study as that one fallen to the lot of the economic theory, the "round-trip" way of development is apparently unavoidable.

The perfect competition model – is a classical sample. The unrealistic model can fairly prove to be extremely creative and heuristic one just owing to its informality (as it turned out to be in fact in the perfect competition model case). And the "revolt" against its admissions doesn't throw the science back, but pushes it forward taking into account the model-made data.

But the expert, as distinct from the theorist, cannot use the unrealistic model "for a time". The demand for the knowledge reality is set much more strictly for him, than for the theorist. He should be adequate to the situation always, every moment of time, here and now, no matter how far could the theorists be from the purely scientific solution of the corresponding problems.

It is obvious that a more realistic course will obligatory prove to be a more complex one. Famous Joan Robinson, the author of the oligopoly theory, once said that economic models could be either simple or realistic. Thus, the use of unrealistic models in the Russian economy should give way to a scientifically grounded approach to the construction of realistic models of economic processes. Here, however, it is necessary to admit that a deep insight into the essence of economic processes is not always achieved due to intense mathematization. The fact that while the "pure" economics was perfecting the mathematical apparatus of the consumer behavior theory, the economic science focused on practice created the foundations of marketing, can serve an example to it. And marketing studied the same consumer behavior, but did it absolutely differently, almost without resting on the concept of marginal utility, but exactly like the firms needed it in their everyday activities.

Thus, the process of training qualified experts will need not only the creation of new textbooks, teachers' retraining, but also a more active involvement of students into research-and-development work in the area of economic processes study, as the approximation of the existing theoretical models to the reality of the present-day economy not always can prove to be well-taken. For the Russian economy there can appear the necessity to create new models.

The research-and-development work at the Finance Academy under the Government of Russian Federation is an integral part of the educational process, the most important factor of strengthening intelligent potential, the ground for a permanent renovation of the methodological support. The quality of research work at the Finance Academy is assured, first of all, by a high level of the faculty professionalism, and also due to the creation of conditions for highly effective research work.

The research work of the Finance Academy students (RWS) is organized, first of all, at the departments with active organizational participation of the Students' Scientific Society and RWS section of the Research Coordination Administration. All together at the Departments of the Academy the work of more than 50 of hobby, task groups and debating-societies is organized. The most large-scale and already traditional ones are the students' scientific activities held within the framework of the annual "Science Week", the International Students' Scientific Conference. The participation of the students in the scientific activities outside the Academy is character-

ized by a high activity. In 2007 the Academy students took part in 32 various scientific actions and won thereat more than 80 different diplomas and grateful letters.

However, not all the students (especially those of junior and middle courses) manifest their wish to take part in scientific actions held by the Academy. In this case the top role in the process of attracting students to research work, in the author's opinion, belongs to the teacher running lectures and practical trainings with the students. It is the very teacher to be in a close contact with the students who can form a stable interest to research work, which is so necessary for a highly qualified specialist.

In the current contest the capacity to study, creativity and thinking flexibility of experts are as important as storage of knowledge. The study at a HEI takes five-six years. How business wants will change during this time, even the business itself doesn't know; that is why one of the concerns of the teacher is to prepare mobile experts with encyclopedic knowledge, to provide them with skills and expertise sufficient to adapt to new labour market demands. HEIs should become the initiators of new activity kinds, the basis for the integration of knowledge into practice, the ground for permanent contacts of the academic and business environments' representatives without reducing the academic training and research work level. In the new generation HEIs the future specialists obtain support from their entrepreneurial initiatives and get experience of their own business-projects management in the course of study. The package of disciplines on a particular speciality is supplemented by a sound training in the area of information technologies and knowledge from linked industries. A wide range of training is also necessary for interdisciplinary research and is profitable for graduates as every once in a while there is experts' overproduction in various economy sectors.

The author of the given article is the research manager of the academic and research Simulation Modeling seminar working at the Department of "Mathematical modeling of economic processes" at the Finance Academy under the Government of Russian Federation. Students discuss the most topical subjects of the Russian economy during panel sessions of the given seminar. The students' best reports are published in collections of scientific papers. With the reports approved at the seminar the students take part in scientific actions outside the Academy. And the most active participants of the seminar get the priority right to choose the training place and the following employment.

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Materials of Conference

MORAL ETHNIC ASPECTS OF DESIGNER'S FORMING

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In each country of the world association there is a specific role in solving the problem of the stable development, which is common to all mankind and is reflected on the national strategy, in accordance with its potential and peculiarities. This is on the question of principle of these strategies to take into account a factor, that for the next generations a priority will be not over-usage of the material good and possession of wealth as an indicator of dignity and rate of the social meaning of man. When satisfying you own material requirements at the most necessary rate such indicators should be intellectual and testament perfection of man, making of the material basis and conditions of the informative analytical, mental cognitive and testament aesthetic development.

Meaning that the paper of the president of the Republic of Kazakhstan N.A. Nazarbaev "In a stream of history"[1] is an important and poly-semantic event in science and cultural life of all the central Asian people. This culturological research in accordance of its content and many methodological aspects discloses a new solving of the problem of over-interpreting and deeper historic learning. A language, memory and culture are an important trilogy of the national mentality and have their over-interpreting in the article.

A term "culture" is of the Latin origin. At first it meant "soil cultivating". Susumo Sato and Khiromitsu Kumamoto in their book "Reengineering of environment" wrote the following: the things made by a man in the agriculture lead to the appearance and affirmation of cyclical interconnection with nature. From the point of view the agriculture can be named as the second nature. It supposed a constant and dynamic interconnection a man and environment. The rural landscapes are enrolled to the nature. As a rule, agriculture is more stable, independent and less aggressive. Contemporary agriculture based on the using of HT is not stable and destructive. It will disappear soon. And only organic agriculture of the ancient times and middle ages can be stable and reliable".

Ancient people went in the direction of the cultural world, when they begin perceiving the nature as an external dependent on them world. According to Portman[2], the animals are closely connected to the nature and can't influence the changes of the environment actively. This leads to the following: the animals and nature are indivisible. Man began dividing from the nature, vice versa. Trying to bend it to his will, he confirmed his independence. Such division lead to the following: a man turned out from the nature being to the cultural one. In the result of this the cultural aspects appeared in his life. An attribute "cultural" became a synonym of the notion "artificial", "made by man". The culture captured the human being forever. In general meaning the culture is understood as all the kinds of transformation activity of man and society and also its results.

From this it follows that the culture is an important characteristics of the social life. That is why it can't be divided from the man as a social being. From the biological point of view a man has just an organism, which has a certain structure, advances and functions. In the process of vital activity a man forms as a cultural historic being. His human characteristics are the result of assimilation of the language, introducing him to the existing in the society value, traditions, learning of the techniques and activity skills, which are common to this culture, etc. The degree of cultural development demonstrates the achievements of the certain historical civilization and indicates it's place of existed, contemporary and future civilization.

Slowness of the requirements of the Kazakh steppe habitants is the following: mental cognitive and testament aesthetic development needs clear sources to give knowledge to students-designers and for the creative implementation of knowledge in the activity.

Such a requirement of the creative implementation concerns first of all a design-process which consists not only in the consumer's requirements of the national design, but in the designers' ones of learning the sources of the Kazakh culture. The specialists of our Republic take part in this process, understanding that a design isn't limited by using this or that style. It is indivisibly connected with the development of new technologies and competition of the goods at the market. It should correspond to the place and time, putting charm into the life, make it comfort and pleasant. At present time the Kazakh design is avanguard and very susceptible to the global vibrations of stylistic concepts. One of the tasks of it is a creation of "today and here" cultural context. As a rule, going into the past as a fact of the real life, a steppe culture became an indivisible part of the national mentality. A phenomenon of "the regional style" almost closed up with "nostalgia of tradition", search of national originality, attempts of connections, "time's connections".

That is why the interest of designers and architects to the culture of steppe nation rises not accidentally. First of all, the parameters and characteristics of this culture are related to the qualities inherent to the design product. Secondly, all the subjects of nomad encampment were selected distinctively during the centuries and perfected from the point of view of their maximal practicability, solidity, firmness, lightness and convenience under the condition of constant changes of placement. These characteristics are very important for the designers' projects. Thirdly, the pieces of art of steppe nations drag up with their force,

primordial purity and unusual rhythmic, for the first sight. Thy cast a spell by the perfect quality of the hand-made work and the quantity of spent energy. Our forbears hang on their necks a little skin rack with a pinch of dried-up wormwood for happiness when going faraway. Who are our forbears?

The researches of the Kazakh people were Turkic-speaking tribes, which had a nomadic style of life. The nomadic habits, home management forming for ages, ancient heatlon ceremonies, elements of shamanism formed a traditionally social culture of national communication, in the content of which a wisdom is reflected. “Adam bol” is a principle of Abai’s philosophy has an important meaning even for today for the moral ethnical education of the youth. It calls to self-development, self-knowledge and self-cleansing. Shakarim’s philosophy consists in knowledge of 3c truths: truth of Belief, truth of Science, truth of Soul-Conscience. It can be regarded as a methodological basic of testament moral education of the rising generation [3].

The following factors influence the moral norms (according to A. Kalybekova):

- daily experience related the activity, way of life, relations;
- moral ethics values succeeded to the previous generations;
- connections of each with micro- and macrosphere of socialization: family, clan, village, city;
- factors of a religious character, their sources are shamanism;
- the contact with other nations: Russian, Mongolian? Kirghiz? Buryat…

Each center and every nation had their own face in the history. It is recognizable. The ancient artists kept the traces of the past. Their art is an object of the search of contemporary science. Coming-to-be of the nation is very long process. This is a real historic phenomenon depending on many reasons. “The traces of ancient people, as it is known, were found in Central Africa and Indo-China. There were 2 centers of the civilization origin. It seems that from this it follows that 2 human races, i.e. Negro and Mongolian. Murad Adgy mentioned in his book “Europe, Turks, Great Steppe” the following: the civilization development lead to the division of people” into the nations. For the first it was connected only with a nature and environment. For instance, the habitants of mountains developed their psychology for ages, their disposition, which differ the ones of coast habitants. And the forest habitants had another culture and disposition. The face of each nation formed for ages; its individuality polished. That is why to draw the nation portrait you need tens of lines, hundreds of color tones” [4].

The archeological data showed that the main part of the territory of Kazakhstan in VII-IV century BC was occupied by the powerful tribe of Saki. Saki occupied the territory of South, East and Central Kazakhstan. Apart of them, there were many others big and small tribes. They also took part in socio-political and cultural events of those times. However, the territory of occupation and certain history of the majority of them are not clear. The written reports are fragmentary and of general character; the archeological material is small.

Lets regard some of them. Archeologist, Professor S.I. Rudenko named nation of Altay Scythian in his books [5]. The first of the European, who informed about Scythian was a Greek writer Gerodot. The texts are composed in accordance with Persian tsar Dariy and Xerks are the written report of our interest. “The most important tribes live on the Evksinsky Ponte, i.e. the place which Daruy visited. In this there is no educated tribe at all. There is no any famous person, expert Scythian Anaharsis. Among all the famous nations only Scythians have the most important for human life art. This is the following: they don’t allow escaping to th enemy that attack their country. Nobody can overtake them, if they don’t allow this. But they don’t have cities, fortifications; they take their houses together. All of them are horse archers. They are busy at cattle-”breeding; their houses are kibitka” [6].

The key moment of the developing of nomad house was invention of the folding and collapsible on links’ frame of the vertical walls. Such constructive design should be regarded as the main in the process of creating classis type of jurta. In favor of this revolution change of the horse construction the actual living space was extended, the length was decreased and the general weight of wooden details too (thanks to the decreased cutting). And the main thing is appearing of the harmonized space for man.

Saki worshiped to the nature forces: sun, wind, thunder and storm, which were represented in the image of God. The Gods were undergone a transformation into the different animals and birds. Popularity of these images in mythology and folklore revived “wild animal style” in the art.

A topic basis of the art of wild animal style were kept from ancient times myths about the origin of people from different animals. An ancestor of clan usually appeared from any wild animal: deer, tiger, steppe eagle, etc. The things with an animal image regarded as holy , played a role of talismans and amulets. A wild animal on the weapon seemed to help in the strike, made it invulnerable and fearless.

This style appeared in the art as a regular expression of attitude of Saki’s tribe as an embodied their mythology in the fine art, as a special sign system for expression of nomad ideology. This style of art creativity in III-I century BC loosed a realistic basis, turned into the ornamental scheme. At the beginning of centuries a bright art of wild animal style disappear at all.

The task for students-designers is complicated by the necessity to understand the art of wild animal style and interpret such themes as ornamentation or

using of traditional constructive, design underlying cause and semantics of regional culture. It is very important that we jumped over the stage of traditional ornaments and appealed to the essence of architecture, i.e. space and objects. However, in general we have the examples of using European architectures language without satiety by the local dialect. One can hope, that the sense of belonging to the world architect cognition and temperament of nomads can result amazingly, i.e. fresh, humane, architecture design lines, striving to the clearness and simplicity, and a gift if tradition.

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Materials of Conference

PECULIARITIES OF TIME PERCEPTION: MULTIDIMENSIONAL PROBLEM

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The problem of subjective time is being developed from different perspectives by many sciences, both natural (Dobrokhotova, Bragina, 1977; Megrabyan, 1972, and others) and humanitarian ones [Elkin, 1962, 1969; Woodrow, 1963, and others]. In psychology the influence of various factors, such as motivation (Budiyansky, 1984), on time perception is being studied, the shots to define the duration of "present" (Molchanov, 1998; Golovakha, Kronik, 1999) are being tried, etc. The problems of subjective time are studied by general and experimental psychology (Rubinstein, 2006; Golovakha, Kronik, 2008), psychophysics (Kahneman, Treisman, Gibbs, 1992; Falikman, Pechenkova, 2001), engineering psychology (Mitina, 1979; Strelkov, 1999), differential psychology (Lisenkova, 1981). And in developmental psychology the fact that the time imagery formation in children is a "necessary precondition of cause-effect and theoretical ideation development, and also the condition providing cognitive activity as a whole" (Andriyevskaya, 1980) is marked out specially. According to Kuznetsov O.N. and his collegues the adequacy of time reflection is necessary for a successful adaptation of a human to the conditions of changing surroundings. However, in spite of the recognition of an important role of subjective perception of time in the comprehension of personality characteristics and great amount of works devoted to this topic the problem cannot be considered a sufficiently developed one.

So, for example, R. Woodrow describes the problem of time perception as a "difficult one to study" (Woodrow, 1963) and points to a frequent inconsistency of results of various works on the given problem. So, many authors show that people underestimate the time for complex tasks execution on the results of some studies, and vice versa – overestimate it on the results of other ones (Rogovin, Karpova, 1985; Kalyagin, 2006).

Besides, when studying time as a psychological phenomenon, investigators use the most diverse terms, among which one can see "time perception", "time reflection", "internal sense", "sense of time", "time imagery", "time judgement", "time memory", "organization of behaviour and activity in time", "time knowledge", "experience of time", "time realization", etc. Some research workers dedicate their papers to the find out if these notions are identical. As a rule, they come to the conclusion that authors have different sense in them and mean the phenomena irreconcilable with each other (Dobrokhotova, Bragina, 1977). And it raises difficulties to the comparison of various investigations' results.

The study of subjective time caused the appearance of conforming tests, wherein a range of operationalized notions, such as, for example, "the definition of time duration" (or "time estimation"), "time measuring", "time reproduction", is used. Let us settle upon them in more detail.

Measuring preset time intervals (1 min, 30 sec, 10 sec, 7 sec is used most often). The experimentalist calls a time interval, which the test person should measure, i.e. specify, when the desired time passes out. The investigator records the time with the help of a stop watch, taking into account the time frame duration accuracy level, and also the frequency and importance of its under- and overestimations.

Verbal estimation of time intervals. Various time intervals are set by a stop watch click or any other method, the test person evaluates their duration verbally.

According to the literary data the estimation of the intervals and their measuring are in converse relations, i.e. the persons, who overestimate the preset time intervals, submeasure them, and vice versa (Mitina, 1977; Lisenkova, 1981, Rogovin, Karpova, 1985).

Time frame reproduction. The test person is offered to reproduce (repeat) an earlier presented interval by means of knocking or any other method. It is considered that a departure from the reference time is connected with the nervous and physiological function of a human, it is an innate characteristic and doesn't change for the whole life period (Elkin, 1962; Tsukanov, 1990).

To study the subject perception of time intervals other methods are also used, the test of Frankenheuser (Kuznetsov and others, 1985), for example, the estimation of a simple sensomotor reaction duration (Mikhailova, 2008), the estimation of accuracy of the reaction to a moving object – RMO (Tochilov, 1970; Mikhailova, 2008), etc. So, one of the early discoveries made when investigating the problem of subjective time in psychology was the detection of a tendency of the test persons either to over- or underestimate time lines, that is referred to their individual features connected with their somatic and nervous function (Lisenkova, 1981). Another subjective time investigation test group is aimed at the measurement of time characteristics within the "fast-slow" diapason. These methods appeal, as a rule, to visual imagery of time ideas. Such methods as pictograms, for example, are used here: the test persons are offered to draw pictures denoting the ideas of "fast" and "slow". A social-perceptual intuitive test (SPIT) (Burlachuk, Morozov, 1999), the stimulating material of which the L. Sondy test pictures serve (Sobchik, 2007): the test person is offered to estimate the time of behavioral reactions of

the people in the pictures judging on their faces' expression.

And, finally, an impressive part of the methods is aimed at the detection of peculiarities of temporal relations reflection by the human in the context of life journey and biography. The methods of simple graphical tests, such as "Time circles" by T. Cottle (Cottle, 1976), together with such time taking autobiographical methods as "Life line" (Golovakha, Kronik, 1993), ZTPI (Zimbardo, 1999) known in Russia as "F. Zimbardo's method on time perspective" and "psychological autobiography" (Burlachuk, Korzhova, 1998), can be referred here.

In the Cottle's graphical test described in the works of A. Kronik and Ye. Golovakha the test person should depict his past, present and future as three circles, the importance and interrelations of these elements are analyzed after that. The questionnaire of F. Zimbardo on time perspective (Zimbardo Time Perspective Inventory) adapted by A. Syrtsova defines the preference of this or that time orientation by the respondents with the help of five factors (Syrtsova and others, 2007).

To study the subjective experience of time in the life scale the "test of semantic associative relations", where the test person is offered to name the associations connected with the ideas of the "present", "past" and "future" (Kuznetsov and others, 1985), is used; the test of semantic differential (the estimation of the present, past and future with the help of specially constructed scales) (Fyodorova, 1978) and other methods allowing evaluating individual differences and experiences of time aspects of the reality are used as well.

Taking into account all the diversity of methods and approaches to the study of subjective time it becomes clear why so different terms are used in the given case. It is evident that to describe the results obtained with the help of various methods and the phenomena found out different notions, which differ from each other qualitatively, are attracted.

Lately psychologists agree that there is no special autonomous mechanism of time accommodation in the human psyche more and more often. We share the opinion of M.S. Rogovin and Ye.V. Karpova, according to which "all the mental mechanisms and processes available in the human are used for this purpose; the same levels of psychic reflection and their operating means, which have been formed evolutionary for the solution of any other accommodation problem, are attracted…" (Rogovin, Karpova, 1984). For, even though physical time can be measured, it is not a stimulus in the usual sense of the word: there is no object, which could have an effect on the receptors of the human perceiving it immediately. That is why the notions of "feeling" and "sense" of time are used when we refer to short time intervals; by the problem amplification and attraction of more protracted time scales the involvement of more complex mental processes and use of the notions conforming to them, such as "realization", "experience" and "judging", is required.

After B.G. Meshcheryakov and V.P. Zinchenko the copy editors of the "Big psychological dictionary" we mean an imagery reflection of such characteristics of real events as duration, course rate and progression by the term of "time perception". We also agree with D.G. Elkin, who showed in his multiyear research that a gradual assimilation of social duration etalons making the system of time lines and scales takes place in the human over the period of his cultural development (Elkin, 1962). It supposes that only upon the condition of all psychic functions maturity an adequate reflection of various time relations is achieved, it playing a great role in different kinds of practical human activity.

In general, when working further in the time perception problem, it becomes apparent that it is extremely complex and studied insufficiently enough, but at the same time the psychological theory and practice demands impose the necessity of its further development. Maybe, it is an integral research of various time perception aspects that will allow connecting the level of different time relations reflection adequacy development with personality characteristics and mental processes.

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ON CHARITY REVIVAL IN PRESENT-DAY RUSSIA

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Charity, as the fundamental principle of present-day forms of social care has an important civilizing meaning – the provision of social equilibrium in the society, and appears, first of all, as the form of self-defence and mutual aid when official powers due to various reasons is not able to solve the problems of the needy.

The charity work in Russia has historical roots, which are connected with the becoming and development of the national identity. At different stages of Russian history the motivation, kinds, methods and degree of social assistance rendering were various. The problems, which non-state voluntary organizations and societies were trying to solve, remained unchanged: they are the "relief" to the needy, custody of the poor, widowed and orphans, moral and financial assistance for the quickest adaptation in conditions of socioeconomic and political reforms, etc.

The role of charity and private initiative grew and is growing again in the state, where insufficient financial assets are provided for social needs, where there is no economic security and the unemployment level is too high (especially in the youth media). Nowadays, the process of transformation of the Russian society is attended by the government and citizens' role alteration and civic initiatives growth. The national social safety net is still unable to solve the set of current and emerging problems. Beyond the administrative-governmental institutions of social protection there arise new philanthropic initiatives, which are aimed at the solution of the social safety problems. As a rule, they are the activity of various voluntary non-state organizations, charity funds and societies.

The present-day charity revival has become a response to the formed socioeconomic situation, which was characterized not only by a considerable impoverishment of a quantity of the population, but also by the government potential weakening. In the Russian society the need to search ways out of the socioeconomic crisis, ways of the social tension remission, and not only by state organs, but also social ones, solving the problem of social assistance for the population, has become actual. The way out of the present-day crisis is seen in the development of charity models maximally conforming to the specificity and traditions of Russian culture and history, taking into account the polyethnicity and polycofessionalism of the society.

The revival and development of charity work should be attended by the National and legal protection. The charity programs and actions will become effective, if they have clear priorities, concentrate on the solution of certain problems, having address character. The virtue of charity is in its mobility, the quickness of response, the variety of aid forms and its individualization. The separation of rights and duties of the state, society and private persons, their interaction and consolidation in the area of social assistance will, certainly, become the most important condition of its efficiency.

Nowadays, various charity funds and organizations called to compliment the efforts of the state in the social and cultural spheres are created in the country. The earlier unclaimed historical experience of the pre-Revolutionary charities', Leagues' and Committees' functioning is especially important for them. In this relation the topicality of historical cognition of charity development in Russia are acquiring a close connection with the formation of the governmental social policy. In connection with the fact that a rich pre-Revolutionary experience of charity was practically lost during the Soviet period of the Russian national identity; the topicality of reconstruction of charity traditions of the past is substantiated by the following tasks: the appearance of practical necessity in support of the needy in the context of the carried out socioeconomic reforms; finding out the problems of development and realization of such social policy, which is able to provide people's life quality growth; the appearance of new reference points of the civil culture, wherein the social support of unprotected layers would take a special place. When studying Russian charity history, its regional aspect, on the one hand, allows imaging the sizes and forms of charity work in the scales of the whole country, and, on the other hand – revealing its special features, which are conditioned by the economic and social development of the region, demographic, national, religious, mental peculiarities of the local population.

The search of a new strategy of the social development faces the problem of compatibility of traditional values and the ethics of new market relations. The charity revival in present-day Russia makes scientific knowledge and theoretical comprehension of the richest experience of social charity actual. The problem attracts attention of historians, sociologists, psychologists, culturologists and other experts, who raise the problem of the formation of a new scientific direction connected with the interdisciplinary studying of the past and present of charity – its historical traditions, modern forms and development prospects.

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