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3rd Central-Eastern European Symposium on Free Nucleic Acids in Non-Invasive Prenatal Diagnosis

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Jessenius Faculty of Medicine
Comenius University, Martin, Slovak Republic

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Dear guests,

We are delighted with the opportunity to welcome you to the the 3rd Central-Eastern European Symposium on Free Nucleic Acids in Non-Invasive Prenatal Diagnosis that is taking place in Martin, in Slovak Republic this year. This interesting symposium is being organized for the third time. The knowledge and the associated technologies have advanced enormously since the first meeting in 2010 in Budapest. In Olomouc in 2012, we had a great opportunity to listen to a number of fascinating lectures. We are looking forward to the latest progress in the field of non-invasive prenatal testing.

The theme of this congress is not only the latest results and improvement sharing. It is also discussion and comparison of NIPT methods and their implementation in several countries. We would also like to facilitate and improve cooperation and communication between clinical specialists, geneticists, technicians and scientists. One of the most discussed topics in NIPT is its implementation in prenatal management schedules, commercialization and ethical issues. We hope all these will be covered in the lectures and subsequent fruitful discussions.

The backbone of every scientific event is well-established expert speakers who are working creatively on their research. We would like to thank all the speakers for accepting our invitation and to all those who have helped us to prepare both the scientific and the social programme. We thank all our partners, mainly our general partner BGI who are making this symposium possible.

We wish you a fruitful time at the symposium, meeting old friends and interesting new people. We hope you will enjoy your stay in Martin. We are looking forward to this exciting scientific event.

On behalf of the whole team
Zora Lasabova
Iveta Svecova

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Noninvasive prenatal testing using fetal DNA in maternal plasma: from chromosomes to genomes to methylomes

Dennis Y. M. Lo

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There is much recent interest in the use of circulating cell-free fetal DNA in maternal plasma for non-invasive prenatal testing (NIPT). In particular, the use of massively parallel sequencing has enabled the non-invasive prenatal detection of a number of chromosomal aneuploidies, subchromosomal deletions, genomewide molecular karyotyping and even fetal whole genome sequencing. Through the use of genomewide bisulfite sequencing of maternal plasma, fetal methylomic profiling has also been accomplished non-invasively. This latter development has exciting implications for research into physiological and pathological processes in development and may have interesting diagnostic applications.

PL-2

Cell-free DNA screening for aneuploidy in public health

Howard Cuckle

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For women who present before 13 weeks gestation the traditional screening protocols have a high model predicted Down syndrome detection rate for a 3% false-positive rate: Combined test 82%, with additional ultrasound markers 91%, Contingent screening with 10-20% having further markers 89-92%, and having both first and second trimester markers 91-96%.

Maternal plasma cell-free (cf)DNA screening yields much better results. From a meta-analysis of large retrospective studies in high risk pregnancies the Down syndrome detection rate is over 99% and the false-positive rate under 0.2%.

Thus, whilst cfDNA testing is not diagnostic, it is increasingly used as a secondary screening test for women found to be at high risk following traditional screening protocols. A positive result will increase risk by a factor of more than 600-fold and a negative result will decrease it about 150-fold. The results for Edwards syndrome are similar but poorer for Patau syndrome, Turner syndrome and the other sex chromosome abnormalities now included in commercial tests.

Results from prospective studies are more difficult to interpret but suggest that performance would be similar, or even better, when cfDNA screening is carried out in the general population. However, current costs of the test preclude completely replacing traditional screening by routine cfDNA screening in public health programs. The estimated cost of avoiding each additional Down syndrome birth missed by traditional screening exceeds several fold the lifetime societal costs of an affected infant.

A compromise solution is to extend the secondary use of cfDNA to include those with borderline as well as positive traditional screening test results. This form of contingent screening using the Combined test and regarding about 10-15% as borderline achieves a high Down syndrome, very few screening false-positives and a favourable cost-benefit equation.

Contingent cfDNA screening could be further improved by extending the Combined test to include additional serum and ultrasound markers. Another improvement would be to estimate from the Combined test markers, the risk of all aneuploidies detectable by cfDNA screening, rather than Down syndrome alone.

One practical difficulty with cfDNA screening is the relatively high proportion of tests which fail, in most cases because of a low fetal fraction. In such cases a second sample is usually drawn but the test is even more likely to fail than in first samples. One of the reasons for a low fetal fraction is reduced placental volume or function, which occurs more often with aneuploidy. Hence cfDNA test failures are at higher than average risk of aneuploidy and might therefore be considered candidates for invasive testing or at least deeper cfDNA sequencing.

In a public health context the optimal use of cfDNA screening for aneuploidy will needs to take into account various other factors. These include the use of traditional screening markers to detect other disorders (eg NT and cardiac abnormalities) or pregnancy conditions (eg PAPP-A and pre-eclampsia), and the use of invasive prenatal diagnosis to detect micro-deletion and -duplication syndromes.

PL- 3

Cost-benefit analysis using different strategies

Howard Cuckle

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PL-4

Non-invasive EXamination of Trisomy (NEXT) Study: Directed cell-free DNA analysis versus 1st trimester combined screening for trisomy 21 risk assessment in a large routine pregnancy population

Mary Norton, Herb Brar, Bo Jacobsson, Geeta Swamy, Angela Ranzini, Mark Tomlinson, Louise Laurent, Leo Pereira, Howard Cuckle, Jean Spitz, Desiree Hollemon, Thomas Musci, Ronald J. Wapner

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Background. Non-invasive prenatal testing (NIPT) with cell-free DNA (cfDNA) has been shown in several studies to be highly accurate for fetal trisomy evaluation in high-risk pregnant women. The performance of NIPT in a routine pregnancy population has yet to be evaluated in a large prospective study.

Objective. To compare the performance of NIPT with directed cfDNA analysis to first trimester combined screening (FTS) for fetal trisomy 21 risk assessment in a general pregnancy population.

Methods. A prospective multi-center blinded cohort study was undertaken comparing the HarmonyTM Prenatal Test, a directed cfDNA test, with FTS using first trimester PAPP-A, hCG and nuchal translucency measurement. Pregnant women with a singleton fetus presenting in the first trimester for routine prenatal screening for fetal aneuploidy were eligible. Participants had both FTS and Harmony testing performed. Women were provided with FTS results as part of routine care. Participants and care providers were blinded to Harmony results, calculated as probability scores. Pregnancies were followed for newborn outcomes. Invasive testing results or neonatal phenotype, with karyotype confirmation in cases of suspected aneuploidy, were used for trisomy 21 identification. Harmony, FTS results and outcomes were reported to an independent data coordinating center. The primary outcome was the comparison of the area under the ROC curve for trisomy 21 test performance of the Harmony and FTS tests.

Results. A total of 18,955 women were enrolled across 38 centers in the United States, Canada and Europe from March 2012 to April 2013. The mean maternal age was 30.6 (range: 18-52) years and the mean gestational age was 12.4 (10-14.3) weeks. As of this submission, all follow-up has been completed.

Final results of this large prospective international study will be presented and implications for the use of NIPT for trisomy 21 risk assessment in the general pregnancy population will be discussed.

PL-5

Contingent screening strategies with and without additional serum and ultrasound markers

Howard Cuckle

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L-1

Extracellular chromosome 21-derived microRNAs in euploid and aneuploid pregnancies

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Background and Objectives. Trisomy 21 is the most common chromosomal aneuploidy in live born infants. Recently, the over expression of chromosome 21-derived microRNAs (miR-99a, let-7c, miR-125b-2, miR-155 and miR-802) in human fetal hippocampus and heart samples from individuals with Down syndrome was observed. Therefore, concentrations and expression profile of extracellular chromosome 21-derived microRNAs were studied to verify their ability to distinguish noninvasively between pregnancies bearing euploid fetuses and those affected with Down syndrome.

Methods. RNA enriched for small RNAs was isolated from plasma samples of 12 pregnant women with high risk of bearing Down syndrome foetuses (median gestation 18.5 wk), 12 women with normal course of gestation and 10 non-pregnant women. MicroRNA transcribed into cDNA using specific stem-loop primer was detected using real-time PCR assay. Simulation experiments using RNA pools of healthy non-pregnant individuals and aneuploid amniotic fluid samples in descending dilution ratio ranging from 1:1 to 1000:1 were used to test the detection limit of the technique for overexpressed chromosome 21-derived microRNAs specific for Down syndrome. The expression profile of the gene encoding microRNA was studied through the relative gene expression using the comparative Ct (threshold cycle) method. Concentrations of individual microRNAs were subtracted from the calibration curves in the course of analyses and expressed as pg of total RNA per milliliter of plasma. Results: Four of the five extracellular chromosome 21-derived microRNAs (miR-99a, let-7c, miR-125b-2 and miR-155) were reliably detected in plasma samples. Simulation experiments revealed the detection limit of aneuploidy at a ratio 100:1 for let-7c, miR-125b-2 and miR-155, and a ratio of 1000:1 for miR-99a. Overexpression of extracellular miR-99a, miR-125b-2 and miR-155 was observed in pregnant women compared to non-pregnant women. Similarly, increased concentrations of extracellular miR-99a and miR-125b-2 were detected in pregnant women than in non-pregnant women. The concentrations and relative gene expression of extracellular chromosome 21-derived microRNAs did not differ between the cohorts of pregnancies bearing euploid foetuses and those affected with Down syndrome. Interpretation & conclusions: Analysis of extracellular chromosome 21-derived microRNAs has no benefit for screening programmes and non-invasive diagnosis of Down syndrome.

ACKNOWLEDGEMENTS

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L-2

Haplotype-assisted accurate noninvasive fetal whole genome recovery through maternal plasma sequencing

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Aims. The applications of massively parallel sequencing technology to fetal cell-free DNA (cff-DNA) have brought new insight to noninvasive prenatal diagnosis. However, most previous studies based on maternal plasma sequencing have been restricted to fetal aneuploidies. To detect specific parentally inherited mutations, invasive approaches to obtain fetal DNA are the current standard in the clinic because of the experimental complexity and resource consumption of previously reported noninvasive approaches.

Methods. Here, we present a simple and effective noninvasive method for accurate fetal genome recovery-assisted with parental haplotypes. The parental haplotype were firstly inferred using a combination strategy of trio and unrelated individuals. Assisted with the parental haplotype, we then employed a hidden Markov model to noninvasively recover the fetal genome through maternal plasma sequencing.

Results. Using ~44X sequence depth against a ~5.69% cff-DNA concentration, we noninvasively inferred fetal genotype and haplotype under different situations of parental heterozygosity. Our data showed that 98.57%, 95.37% and 98.45% of paternal autosome alleles, maternal autosome alleles and maternal chromosome X in the fetal haplotypes, respectively, were recovered accurately. Additionally, we obtained efficient coverage or strong linkage of 96.65% of reported Mendelian-disorder genes and 98.90% of complex disease-associated markers.

Conclusions. In summary, we report an accurate and easy method for noninvasive fetal whole genome recovery by maternal plasma sequencing. An accurate fetal haplotype would enhance the dimensionality of fetal variation detection in prenatal diagnosis/screening and promote the development of fetal medicine. Our results indicate

the potential of using sequencing technology in prenatal diagnosis, and they should accelerate the application of sequencing technology in clinical trials.

L-3

Our experience with non-invasive aneuploidy testing using NGS method

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The Prenascan is a project that was introduced in the Czech Republic in collaboration with Healthy BGI Europe. This is a non-invasive prenatal test for trisomy of chromosomes 13,18, 21 with the possibility of sex determination, including the determination of sex chromosome defects .

Prenascan is one of the screening methods that complement existing biochemical analysis. This test is performable between 10th and 21st week of pregnancy after genetic counseling. Currently, the assay can be used for multiple pregnancies.

The method is based on the evaluation of fragment's relative abundance of cell-free DNA in maternal plasma using whole-genome sequencing and subsequent bioinformatics data analysis. The amount of free fetal DNA (cffDNA) from apoptotic cells trophoblast is between 5 and 10% and according to the stage of pregnancy.

From October 2012 to December 2014, the number of tested patients was 1263. Positivity was found in 22 cases, false positivity in 4 cases. False-positive finds were tested by amniocentesis or chorionic villus sampling with normal results.

L-4

Non-invasive prenatal testing of trisomy 21 and 18 by DNA massively parallel sequencing (MPS) for maternal plasma DNA in twin pregnancies

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Aims. With the increasing trend of twin pregnancies in the last decades, the need to seek an accurate approach for noninvasive prenatal testing becomes urgent. Our study aimed to evaluate the performance of noninvasive prenatal testing of Trisomies 21 and 18 in twin pregnancies by maternal plasma sequencing.

Methods. Pregnant women with live twin fetuses were recruited, with careful pre-test counseling, from six hospitals during April to December 2012 for this study. Written informed consent was obtained from each participant. All MPS-based tests were performed prior to the recording of karyotyping information and the sequencing lab was blinded. All samples performed karyotyping according such indications as follows: i) positive results in maternal serum screening tests, ii) increased nuchal translucency (NT), iii) absence of fetal nasal bone, iv) abnormal ultrasound findings in second trimester, v) twin pregnant women by IVF. 5 mL peripheral venous blood sample was obtained 30 min before invasive procedures. Maternal plasma was isolated within eight hours by a double-centrifugation protocol and stored at -80 °C. DNA was extracted from 600 uL maternal plasma and sequenced on Illumina HiSeq 2000 platform. For each sample, the report was delivered within 12 days after blood sampling.

Results. Totally 128 samples were collected and were treated as real clinical samples, then processed to sequencing immediately after sample collection without delay. The maternal age ranged from 21 to 40 years old and the gestational age from 11th to 27th weeks. With the same pipeline for the singleton pregnancy, we correctly identified two cases with discordant fetal Trisomy 21 and one case with discordant fetal Trisomy 18. The rest 125 samples were classified as negative. Compared with the results of full karyotyping, the estimated sensitivity and specificity for trisomies 21 and 18 were 100%.

Conclusions. Our study suggested that NIPT for Trisomies 21 and 18 by maternal plasma DNA sequencing is of high sensitivity and specificity in twin pregnancies. It has the potential to be used as an alternative option of prenatal test for twin pregnancies.

L-5

Development of a novel, digital PCR-based non-invasive screening method for numerical chromosomal abnormalities

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Aims. Recent improvements in the non-invasive prenatal screening for the numerical chromosomal abnormalities resulted in the development of commercially available cell-free DNA-based tests performed on next-generation sequencing platforms. Unfortunately, due to the applied technology these tests are expensive and are not available for a wide range of pregnant women. In this study we aimed to develop a digital PCR-based alternative platform for the non-invasive screening of the numerical chromosomal abnormalities.

Methods. We have enrolled high risk pregnant women (n=218) into our study who were ahead of invasive procedures and thus cytogenetic investigations. After informed consents were obtained, peripheral blood was taken from all participants. Serawas separated from the blood cells in all samples, cell-free DNA was isolated and digital PCR was carried out on all samples. We have developed a chromosome dosage approach and compared the copy numbers of small fragments of chromosome 21 to chromosome 1.

Results. The parallel running cytogenetic investigations identified 6 women carrying Down syndrome fetuses, all the other samples were negative for Down syndrome (n=212). The digital PCR results were in good agreement with the results of the cytogenetic investigations. To determine its specificity and sensitivity we are now performing large-scale screening of pregnant women.

Conclusions. In the future we plan to introduce this method for the screening of other numerical chromosomal abnormalities. The newly developed digital PCR-based dosage approach method may serve as a new, cheap, fast and reliable platform and a good alternative or pre-screening method before next-generation sequencing-based tests or invasive investigations.

L-6

Noninvasive RhD genotyping in eastern Croatia-first steps

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Aims. In order to further improve antenatal care in our region, we started pilot study with the final aim to implement method for noninvasive fetal RhD genotyping from maternal plasma.

Methods. Our investigated cohort consisted of 100 serologically RhD negative pregnant women. They were randomly selected and between 10th and 33rd week of gestation (median 14).

In first part of our investigation, 30 plasma samples from RhD negative women carrying male fetuses were used for the selection of optimal method for cell free (cf) fetal DNA extraction. We compared 4 different spin column methods: QIAamp DNA Blood Mini Kit (DBM), QIAamp DSP Virus Spin Kit (DSP), QIAamp MinElute Virus Spin Kit (MEV) (all 3 manufactured by QIAGEN), Nucleo Spin NS Plasma XS (NSP) (MACHEREY NAGEL). Slight modifications of the manufacturer's protocols were applied. Yields of extracted cfDNA were evaluated by quantification assays for total cfDNA (hTERT gene) and fetal cfDNA (SRY gene). After the statistical comparison of the yields obtained and taking into account other factors such as price and availability of columns in Croatia, we decided to continue our study by using QIAamp MinElute Virus Spin Kit for all the remaining plasma samples (70).

Second part of the study focused on determination of fetal RhD status. All extracted cfDNA samples were tested for the presence of exons 5 and 7 of the RhD gene. SRY gene was chosen for monitoring of presence and quantification of fetal male cfDNA and hTERT gene was used to quantify total cfDNA. Amplification data were collected and analyzed with an ABI Prism 7000 SDS instrument (Applied Biosystems). Data regarding the fetal gender and serological RhD results were collected after the delivery. Specificity and sensitivity of the noninvasive fetal RHD status determination was calculated.

Results. In the first part of our study, results were as follows: median (range) in ng/ul of total cfDNA for DBM 0.04 (0.0133-0.124), for DSP 0.07 (0.0252-0.2120), for MEV 0.09 (0.039-0.284) and for NSP 0.008 (0.0008-0.102); median (range) in ng/uL of fetal cfDNA for DBM 0.00134 (0-0.005), for DSP 0.003 (0.0007-0.01), for MEV 0.003 (0.0006-0.02) and for NSP 0 (0-0.004). Statistical comparison between the extraction methods used was performed using Friedman's test and *post hoc* Wilcoxon's test (P values<0.05 were considered significant). Statistically significant differencies in the yield of total cfDNA were established between DBM, DSP, MEV, NSP (Chi-square=115.427, P<0.001), and post hoc analysis determined significant differences between all pairs the methods (P<0.001 for all pairs, respectively). The difference was also found between fetal DBM, DSP, MEV, NSP (Chi-square=85.791, P<0.001), and *post hoc* analysis determined significant differences (P<0.001) between all pairs of the methods, except between DSP and MEV.

In the second part of our study, fetus was proclaimed RHD positive when we obtained signals in 2 out of 3 amplicons for each of tested loci or 3 signals from one locus. Determination of fetal RhD status was successful in 99 out of 100 samples. One sample tested falsely negative. Duplex PCR for exons 5 and 7 showed sensitivity of 98.5% and specificity of 100%.

Conclusions. Noninvasive fetal RHD genotyping using spin column based extraction and real time PCR can be implemented as routine diagnostic practice even in small centers. In order to improve sensitivity of the result, additional regions of RHD gene should be included in the assay.

L-7

Circulating C19MC microRNAs in preeclampsia, gestational hypertension and fetal growth restriction

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Signs of maternal inflammation which appear to be present in normal pregnancies at term are exaggerated in preeclampsia and fetal growth restriction, major complications responsible for maternal and perinatal morbidity and mortality. A hypoxic environment induces excessive trophoblast cell death and increased shedding of placenta debris into the maternal circulation. The objective of the study was to identify the profile of circulating C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525 and miR-526a) in patients with established preeclampsia (n=63), fetal growth restriction (n=27), and gestational hypertension (n=23). We examined the correlation between plasmatic concentrations and expression levels of microRNAs and the severity of the disease with respect to clinical signs, requirements for the delivery and Doppler ultrasound parameters.

Using absolute and relative quantification approaches, increased extracellular C19MC microRNA levels (miR-516-5p, P=0.037, P=0.005; miR-517*, P=0.033, P=0.028; miR-520a*, P=0.001, P=0.011; miR-525, P=0.026, P=0.01; miR-526a, P=0.03, P=0.034) were detected in patients with preeclampsia. The association analysis pointed to no relationship between C19MC microRNA plasmatic concentrations and expression profile and identified risk factors for a poorer perinatal outcome. However, the dependence between the levels of plasmatic C19MC microRNAs and the pulsatility index in the middle cerebral artery and the values of cerebroplacental ratio was demonstrated. The study brought interesting finding that the up-regulation of miR-516-5p, miR-517*, miR-520a*, miR-525 and miR-526a is a characteristic phenomenon of established preeclampsia.

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L-8

Clinical implementation of noninvasive prenatal testing using cell free DNA for fetal aneuploidy

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Noninvasive prenatal testing (NIPT) using cell free DNA for risk assessment for the common autosomal trisomies has demonstrated high sensitivity and specificity in multiple studies. While NIPT has been introduced and widely adopted around the world, how best to incorporate the test into clinical practice has been an ongoing subject of discussion. In this presentation, the latest peer-reviewed NIPT implementation protocols and results including performance data will be presented. Practical considerations of NIPT integration into routine prenatal care will also be discussed.

L-9

Prenatal trisomy testing on IonTorrent PGM

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Recent studies focused on utilization of next-generation sequencing (NGS) technology in non-invasive prenatal detection of trisomy of chromosome 21 (T21) showed high sensitivity and specificity if more than 10

milion of reads per sample were used. For these analyses high-throughput NGS systems are needed. The aim of our study was to test feasibility of usage of NGS protocol with approx. 5 milion of reads per sample and make trisomy testing doable also in laboratories with low-throughput next generation sequencing systems like IonTorrent PGM. Recently published study of similar design reported 100% sensitivity and specificity, but was performed on small sample group. We therefore enlarged the sample group and calculated specificity and sensitivity based on our results.

In our retrospective study high-risk category samples of 74 pregnant women were analyzed by low coverage genomic sequencing on IonTorrent PGM. Among these were 69 euploid samples and 5 T21 bearing fetuses confirmed by previous invasive procedure. The standard fragment library protocol was used in library preparation steps. After alignment of reads the Z-score was calculated.

In non-trisomic and trisomic samples on average 5 650 640 and 6 292 096 mapped reads per sample were used in Z-score calculation, respectively. In non-trisomic and trisomic samples average Z-scores were -0.0017 (SD=1.83) and 7.76 (SD=3.75), respectively. All samples with T21 were identified correctly with Z-scores above 3. In our first round of analysis 3 samples without T21 were false positive as they reached Z-scores 4.02, 5.24 and 8.57. The sensitivity and specificity of the tested protocol in our pilot study of T21 aneuploidy detection were 95.65% and 100%, respectively.

According to our results low coverage sequencing based on IonTorrent PGM sequencing is suitable for non-invasive prenatal trisomy testing, however, a further study based on data from larger sample cohort especialy with more samples with confirmed T21 is needed for more precise calculation of sensitivity and specificity of tested protocol. Currently the study continues on greater group of samples and alternative bioinformatic analysis tools are tested to optimize the analysis.

This work is a result of implementation of project REVOGENE – Research centre for molecular genetics (ITMS 26240220067) supported by the Research & Development Operational Programme funded by the ERDF.

L-10

Increased cell-free fetal DNA in preeclampsia - cause or consequence? Data from animal experiments

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Cell-free fetal DNA in maternal circulation is very useful source of genetic information for non-invasive prenatal diagnosis. On contrary, little is known about its role in the pathogenesis of pregnancy-related diseases. We hypothesized that increased fetal DNA present in preeclamptic women might be the cause rather than the consequence of the disease. The aim of our study was to prove our hypothesis in animal experiments.

In two widely used animal models preeclampsia induced by lipopolysaccharide injection or inhibition of nitric oxide synthase did not increase fetal DNA in pregnant rats assessed using SRY-targeted real time PCR. In an independent experiment, saline or fetal DNA was injected (300 μg i.p. daily for 5 days) during the first, second and third week of gestation. No differences in blood pressure were found between the experimental groups and the control group. When fetal DNA was injected during the third week of gestation, maternal microalbuminuria and fetal hypotrophy was found.

Based on our results induction of preeclampsia in animal models does not increase fetal DNA. Higher fetal DNA in maternal circulation in preeclamptic pregnancies seems not to be a consequence of preeclampsia. Injections of exogenous fetal DNA into pregnant rats induced preeclampsia at least partially suggesting that fetal DNA is one of the causes rather than a consequence of preeclampsia. These results might be of importance for further studies on the etiology and pathogenesis of preeclampsia.

L-11

Noninvasive prenatal RHD and KEL genotyping using TaqMan Real time PCR and by capillary electrophoresis minisequencing

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Introduction. There are two reasons for establishing a methodology for non-invasive determination of RHD and KEL genotypes in early pregnancy. To identify fetuses which are at risk of hemolytic disease of fetus and newborn by alloimmunized pregnant women and to prevent alloimmunization during pregnancy.

Aim. RHD and KEL noninvasive determination of the fetal genotype from fragmented fetal DNA in maternal plasma RHD and KEL negative pregnant women using TaqMan Real Time PCR and minisequencing (SNaPshot). Project is supported by IGA MZ CR: NT12225.

Material and Methods. Allelic discrimination by multiplex TaqMan Real Time PCR involves amplification and detection of RHD specific region and KEL 1/2 single nucleotide polymorphism and region of the AMELY gene as an internal amplification control. The methodology was primarily tested on DNA samples isolated from peripheral blood leukocytes of RHD negative/positive, k/k, K/K and k/K samples. 2) SNaPshot: Determination of the sensitivity threshold KEL calibration was performed using a dilution series that were prepared using plasma and cellular DNA KEL negative homozygote (kk) and heterozygote (Kk). It was tested a total of 141 random samples of cell free fetal DNA isolated from maternal plasma in the first trimester.

Result. Both approaches, the parallel and the internal controls are suitable for Non-invasive RHD genotyping. KEL male genotypes determined from leukocyte DNA samples were distinguishable, but there was fluorescence background of non-specifically bound probe. By TaqMan assay, there was not able to distinguish between false positive fluorescence background and fetal DNA admixture. By SNaPshot, on the basis of dilution series it was possible to detect less than 0.78% admixture of K allele corresponding DNA concentration of 0.04 ng/mL. Seven fetuses with allele K were found in 113 of appropriate samples (mother kk), which corresponds to about 4-5 % of the population frequency. It was not possible to determine the KEL genotype of the fetus at 8 samples due KEL heterozygous genotype of the mothers.

Conclusion. TaqMan Real Time PCR is suitable for cff DNA RHD genotyping but method is not able to clearly distinguish the fetal KEL genotype. Capillary electrophoresis minisequencing is more appropriate for the detection of K fetal allele from cffDNA in maternal plasma. Our result will be confirmed on DNA sample after delivery.

L-12

Let-7c as potential maternal serum miRNA biomarker in fetal congenital heart defects

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Aims. Congenital heart defects (CHD) are the most common group of birth defects with incidence of 5-8/1000 live birth. It accounts for 40% of perinatal death. Fetal echocardiography is the most widely used screening tool for CHD. There are a few biomarker for the screening beside the ultrasound, like beta-hCG and PAPP-A. The miRNAs are short, non-coding RNA molecules that play important role in regulation of eukaryotic gene expression. The focus of the current research is to examine free fetal nucleic acids in maternal circulation and to investigate the role of miRNAs related to heart development of the fetus.

Method. Peripheral blood samples were collected from 53 women, 27 of them had healthy and 26 had fetuses with congenital heart defects. miRNA was isolated from maternal serum and the concentration was measured by Nanodrop spectrophotometer. By using Gene Ontology and miRBase databases we searched for those miRNAs which are present in plasma and are associated with congenital heart defects. These criteria were fulfilled by *let-7c* miRNA on chromosome 21. RT-PCR was carried out to determine the *u6* and *let-7c* miRNA's expression.

Results. There was no difference between the miRNA concentrations in the two study groups (6.05 ng/μL vs. 5.36 ng/μL), while we found significant difference in the *let-7c* concentration among the affected and control group (1.12±3.19 ng/μL vs. 0.00047±0.00083 ng/μL; $P<0.0001$).

Conclusions. Fetal-derived miRNAs are part of the free nucleic acids found in maternal plasma, expression studies reveals new opportunities for congenital heart defect research and diagnosis. According to our study *let-7c* seems to be a potential biomarker for fetal CHD.

L-13

Comparing performance of small NGS platforms in non-invasive aneuploidy screening test

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Testing for fetal aneuploidies has been a major research focus since Dennis YM Lo et al. discovered that cell free fetal DNA is present in plasma of maternal peripheral blood in 1997. No suitable technology was available at that time to allow such detection. In 2005, the first instrument using next generation sequencing (NGS) technology was introduced. One of key aspects of NGS, the massively parallel analysis of singular DNA fragments, was recognized by leading groups in non-invasive prenatal diagnostic research as suitable for detection of chromosomal aberrations. Since 2010, small benchtop NGS instruments allowed that a larger scientific community benefits from the NGS technology. However, small systems have limits and they are generally considered insufficient for non-invasive aneuploidy testing.

We used 2 benchtop NGS systems to sequence a set of samples of plasma DNA from pregnancies – LT Ion PGM and Illumina Miseq. Low coverage whole human genome paired end fragment library sequencing was used. Eight samples were multiplexed on Miseq and one sample was run on Ion PGM in each run. Data from both systems in the form of fastq files were analyzed using an in-house pipeline based on previously published workflows. Seventeen samples of pregnancies with euploid fetus were used as the training set. Testing set represented 23 euploid samples and 4 samples defined as trisomy 21. Z-score above 3 was considered positive for trisomy 21 according to published data. No false negative result was obtained on both systems. Five false positive results were identified on Ion PGM and 1 false positive on MiSeq. Average Z scores for euploid pregnancies were 1.35 (SD 3.08) on Ion PGM and 0.04 (SD 2.00) on Miseq. Average Z scores for T21 pregnancies were 10.58 (SD 4.95) on Ion PGM and 6.15 (SD 0.44) on Miseq. The same false positive sample from MiSeq system was false positive on Ion PGM.

We believe that the use of benchtop NGS systems is feasible for trisomy testing in laboratories with lower sample throughput. According to our first experience the Miseq system appears to have a better routine potential. A larger sample set is needed to better estimate the sensitivity and specificity of our approach. Some of the samples may require clinical follow-up.

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L-14

Methods of cffDNA proccesing and analysis determine your results

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L-15

Epigenetic approaches for the NIPD of trisomy 21 in the small laboratory

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Aims. As foetal DNA represents a minor fraction of maternal plasma DNA, the use of foetal DNA for the non-invasive prenatal diagnosis (NIPD) is still extreme challenging. Epigenetic approaches of trisomies (NIPD) have been already published. Design of our study was based on the assumption of two differentially methylated regions (DMRs), from them one hypermethylated lying on chromosome 21, EP6, and the second one hypermethylated not lying on chromosome 21, respective CYP2E1 gene. This gene is totally silent in fetuses and transcriptional activation begins immediately after birth due to demethylation of CpG residues within the first exon and first intron.

Methods. Noninvasive prenatal diagnosis by MeDIP (methylated-DNA based immunopreciptitation) real-time qPCR. Five microliters of peripheral blood were collected from each participant (in 16.-19. week of gravidity) to EDTA tubes. Twelve pregnant women carrying healthy fetuses and 12 pregnant women with trisomic fetuses were involved into the study. Immediately after sampling, maternal peripheral blood samples were centrifuged at 1,600 g for 10 min at 4 °C and the plasma portion was re-centrifuged at 16,000 g for 10 minutes to minimize any additional release of maternal DNA. Circulating foetal DNA

was extracted from 0,8 mL of maternal plasma using the QIAamp DNA Blood Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. Each DNA sample was eluted into 25 μL of sterile, DNase-free water and whole eluate was used for the enrichment of methylated DNA by using the Methylated DNA IP kit (ZymoResearch). The CpG island prediction in CYP2E1 gene (NG_008383.1) was performed in Methyl Primer-Express v.1 (Applied Biosystems) and dual-labeled probe with primers were designed in the IDT Tools *(www.idtdna.com).* Annealing temperature for real-time PCR was set for both real-time PCR reactions the same, 58 °C and reactions were performed by using LightCycler HybProbe chemicals (Roche). Finally, Ct values of hypermethylated CYP2E1 gene were used to normalize the qPCR data sets.

Results. Statistically significant differences were obtained for ΔCt (Ct_{CYP2E1}-Ct_{EP6}) between plasma samples from mothers carrying health and trisomic fetuses. Compared groups of maternal plasma differed on 0.1% level of significance (T-test, P<0.001).

Conclusion. Our data indicate that DMR locuses EP6 and CYP2E1 connected with real-time PCR technology may be potentially useful markers for non-invasive prenatal detection of foetal Down syndrome (DS). As the study was performed only on small sample size, a large scale study within different ethnic populations seems to be necessary to carry out with the aim to achieve greater precision in risk estimation of DS. This methodology presents a favourable approach to the issue of NIPD as the eventual solution in small laboratories.

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L-16

Non-invasive prenatal diagnosis of FGFR3 gene-related skeletal dysplasias using cell-free fetal DNA

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Background. Achondroplasia is one of the most common forms of chondrodysplasia postnataly characterised by short limbs, macrocephaly with a prominent forehead and flattened nasal bridge. The majority of cases is sporadic and results from a *de novo* mutation c.1138G>A in *FGFR3* gene (98% cases). Prenatal ultrasound examination of achondroplasia often fails but sometimes it is suspected during the last trimester mainly by evidence of short limbs. Thanatophoric dysplasia (TD) is a lethal form of skeletal dysplasia. In TD type I the femora is curved and milder form of craniosynostosis is observed while in TD type II the femora is straight and a skull often has a cloverleaf shape. Almost all confirmed cases of TD carry one of the mutations in the *FGFR3* gene. Since both achondroplasia and TD have dominant inheritance patterns and mutations occur predominantly *de novo,* non-invasive prenatal diagnosis using cffDNA and targeting specific *FGFR3* gene regions is possible due to absence of the mutant allele in the maternal genome.

Aim. Our aim is to improve prenatal diagnosis of achondroplasia and other *F GFR3* gene- related skeletal dysplasias by implementation of cell-free fetal DNA (cffDNA) analysis.

Methods. For non-invasive prenatal diagnosis of *FGFR3* gene-related dysplasias cffDNA was extracted from maternal plasmas of pregnancies with increased risk of achondroplasia or TD between 17 and 37 gestation weeks. For c.1138G>A mutation detection we used modified protocol using PCR combined with restriction analysis and followed by fragmentation analysis. Recently, we also developed targeted assay which allows us to analyze selected *FGFR3* gene regions including critical achondroplasia/TD mutations with very high sensitivity using ultra-deep next generation sequencing (NGS).

Results. Nineteen cases at risk of achondroplasia or TD were scanned in our centre. In two pregnancies (27 and 36 gestation week) mutation c.1138G>A specific for achondroplasia was detected in cffDNA. Both cases exhibited reduced ossification and shortening of long limbs, slight hypoplasia of the chest and frontal bossing. Mutation was confirmed by genomic fetal or newborn DNA analysis. These two cases plus one case with TD (mutation c.1118G>A) which was previously detected by classical invasive techniques where used for validation of targeted ultra-deep NGS assay of *FGFR3* specific regions. First experience with this approach will be presented.

Conclusion. Non-invasive prenatal diagnosis of achondroplasia and other *FGFR3* gene- related skeletal dysplasias using cffDNA is a safe alternative approach to invasive testing. Accurate diagnosis is important for parental counselling as well as for clinical management in time of delivery and for the newborn with achondroplasia. It has also an important psychological aspect since the family can be prepared for the child with this condition.

L-17

Is sequencing of free plasma DNA feasible for preeclampsia detection?

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Preeclampsia represents a leading cause of maternal deaths in Europe. Despite all scientific effort, there is no effective screening test allowing the prediction of onset or severe outcomes to date. Among other reasons, the lack of such test can be attributed to limited understanding of the etiology of preeclampsia. It has been shown that increased cell free fetal DNA concentration can be detected in preeclampsia patients before the manifestation of first clinical symptoms. In our previously published work we hypothesized, that the increase level of fetal DNA fraction could be caused by the insufficient capacity of DNases to clear fetal DNA from circulation. If this was the cause of preeclampsia, we expect to detect not only larger fraction of fetal origin in total plasma DNA but also longer fetal fragments. The aim of our study was to determine, if DNA fragments of fetal origin are longer in preeclampsia pregnancies. We used low coverage whole human genome paired-end massively parallel sequencing of fragments prepared from DNA isolated from maternal peripheral plasma samples. We looked at Y chromosomal fragment sizes based on mapping distances of paired-end reads for male bearing normal and preeclampsia pregnancies. We used a set of clinically well defined samples, 20 samples from women with normotensive pregnancies and 20 samples from women with preeclampsia. The preliminary analysis of partial results showed no significant difference in sizes of fetal fragments between normal and preeclampsia pregnancies. This work is a result of implementation of project REVOGENE – Research centre for molecular genetics (ITMS 26240220067) supported by the Research & Development Operational Programme funded by the ERDF.

L-18

Plasma DNase activity in preeclampsia

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Several clinical studies have shown that fetal DNA is higher in patients with preeclampsia even before the onset of symptoms. Although it is not clear whether this is part of the cause or consequence of the disease, we hypothesized that it might be due to decreased DNase activity in preeclamptic pregnancies.

Blood was collected from 57 patients with preeclampsia and 53 healthy pregnant women in two independent cohorts. The groups did not differ regarding age, fetal sex or gestation week. In addition, in an animal experiment fetal DNA was injected into pregnant rats during the last third of gestation. Plasma DNase activity was measured in samples from human and animal studies using an ELISA based assay (DNase Activity, ORGENTEC Diagnostika GmbH, Germany).

The DNase activity was significantly higher in preeclamptic women in comparison to controls in the first as well as in the second cohort ($P<0.05$; $P<0.001$). The mean difference was 5.38% and 15.18%, respectively. In the animal experiment injection of fetal DNA into pregnant rats did not affect the overall higher plasma DNase activity.

According to our knowledge this is the first report of increased plasma DNase activity in preeclamptic women. These results are in contrast to our previous hypothesis. Results from the animal experiment have to be taken with caution due to much higher plasma DNase activity in rats. However, the lack of effect of fetal DNA on plasma DNase activity suggests that there might be other important factors such as extracellular DNA protected with histones or membranes.

L-19

Non-invasive prenatal fetal RhD genotyping using maternal plasma

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Immunoprophylaxis with IgG anti-RhD is a standard prevention of hemolytic disease of the fetus and newborn. Using non-invasive prenatal testing with utilization of cell-free fetal DNA (cffDNA) isolated from maternal plasma the necessity of immunoprophylaxis can be predicted with high precision. This possibility enables the prophylaxis to meet criteria of current trend in personalization of healthcare as it allows targeted IgG anti-RhD prophylaxis in RhD-positive pregnancies only.

Aim. The aim of our study was to optimize and validate noninvasive fetal RhD blood group genotyping from maternal plasma in our laboratory.

Following manual extraction of cffDNA from maternal plasma, real time PCR was used for the detection of sequences of *RHD* gene (sequences of exon 4, exon 5, exon 7 were used as targets) and sequences of androgen receptor gene (used as positive control of the presence of DNA).

Results. Results of cffDNA analysis from maternal plasma were compared with results from analysis of amniotic fluid samples and/or phenotype of the child after birth.

Alltogether 56 samples of cffDNA were analyzed up to now. In 17 and 12 of these noninvasive test results were in full concordance with results of analysis of control samples originated from amniocentesis and neonatal phenotypes, respectively.

Conclusion. Methodology for non-invasive prenatal determination of fetal RhD status was successfully implemented in our laboratory. In our sample group 18 pregnant women with RhD negative fetuses (approx. 32%) who did not required IgG anti-RhD immunoprophylaxis were identified.

L-20

Our experience in non-invasive second and first-trimester RhD genotyping

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Introduction. Hemolytic disease of the newborn occurs in the case of fetomaternal incompatibilities within any number of different erythrocyte antigen systems, including the RhD, RhCE, Kell, Kidd and Duffy. The deletion of *RHD* gene is the most common form of D-negativity in Caucasian population. The elucidation of the molecular basis of the blood group systems allowed the development of PCR-based assays for blood group typing. The discovery of fetal DNA free circulating in maternal plasma during pregnancy enabled the development of non-invasive *RHD* assessment of the fetus. This method became a new diagnostic tool with real impact on clinical settings. In our study, we focused on noninvasive determination of *RHD* genotypes and its correlation to phenotype in *RHD* negative pregnancies in homogenous population of D-negative women from Northern Slovakia.

Methods. The DNA extraction from peripheral blood of RhD-negative pregnant women was performed using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), cff DNA was isolated from 500 μL (method I) or 1000 μL (method II) of maternal plasma using QIAamp DSP Virus Kit (Qiagen). The realtime PCR was performed using TaqMan genotyping with *RHD* exon 7 and 10 specific primers and probes in triplicate. We used *HBB* for total and *SRY* for fetal DNA as controls. Sample positive in all three triplicates were considered as positive. Sample negative in all triplicates were considered as negative. Samples positive in one triplicate were considered as inconsistent. The *RHD* genotype was compared to the immunologically assessed phenotype of the newborn. Student *t*-test was used for statistical evaluation.

Results. The methods I and II were applied on plasma samples obtained from 37 and 20 pregnant RhD- women in II. trimester, respectively. The plasma testing for the presence of *RHD* Ex7 and Ex10 sequences in RhD- as well as *RHD* Ex 7 and Ex 10 negative women revealed that all with exception of two as positive evaluated samples were positive in all three triplicates. For the method I, the Ct intervals for *RHD*Ex7 and Ex10 were from 35.44 up to 41.67 and 34.26 up to 43.07, respectively. For the method II the Ct intervals for *RHD*Ex7 and Ex10 were from 35.09 up to 39.14 and 37.15 up to 40.7 respectively. Comparison of Ct means for exon 7 were 39.98 and 36.35 by Student t-test showed significant improvement of the method ($P<0.0001$). The mean Ct values for exon 10 were

38.99 and 38.47 (P=0.32). All 33 *RHD* positive newborns were confirmed immunologically. From 24 immunologically RhD negative newborns were 23 negative and one false positive. Testing in 28 I. trimester samples showed 100% specificity and sensitivity.

Conclusion. We used an upstream step of maternal DNA testing which excluded the RhD positive blood samples. Here we demonstrate our first experience in non-invasive *RHD* genotyping in a homogenous population from the region of Northern Slovakia. The results for method I in second trimester showed that the testing using the *RHD* exons 7 and 10 specific probes showed 100% sensitivity and specificity. Lowering of the average Ct in the method II leads to 1 false positive result in second trimester but showed 100% specificity and sensitivity compared with RhD standard testing on first trimester samples.

L-21

Potential use of miRNA as universal fetal marker

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Introduction. Circulating microRNA molecules (miRNAs) are currently studied as potential diagnostic markers in many physiological and pathophysiological conditions such as oncological diseases, heart diseases and pregnancy. The aim of our study was to determine if circulating miRNAs can be used as potential universal fetal marker in NIPT.

Methods. Circulating miRNAs were isolated from plasma of pregnant women and miRNA samples were divided into two groups depending on fetal gender. Firstly, expression levels of 91 miRNAs were established in three female and three male samples by two-step RT-qPCR. Five miRNAs (miR-378, miR-122, miR-20b, miR-320a, miR-320b) whose expression levels significantly differ between two groups were chosen for further analyses. Subsequently, expresion levels of five selected miRNAs were evaluated in 14 female and 15 male samples by two-step RT-qPCR. Differences between groups were tested using Student t-test with P<0.05 was consider as statistically significant.

Results. After the first part of this study where panel of 91 miRNAs were investigated, five miRNAs significantly differ in their expression levels between two sample groups. However, there were no significant differences in expression levels of five selected miRNAs between sample groups, when expression levels of these miRNAs were determined in a greater number of samples.

Conclusion. Our study showed, that none of evaluated miRNA molecules in this work is not suitable as the universal fetal marker. However, since our work addressed only limited number of miRNA molecules, for determination if circulating miRNAs can be used as universal fetal marker, further studies are needed.

L-22

Ion Torrent/Ion Proton-from the unique technology of DNA sequencing to use in diagnostics

Ondrej Holena

Life Technologies, Prague, Czech Republic

Torrent technology has pioneered an entirely new approach to sequencing. Instead of using light as an intermediary to sequence DNA, Ion Torrent uses semiconductor technology and simple chemistry. Ion Torrent translates chemical signals into digital information, much as a CMOS chip translates photons into pixels. Just as the microprocessor enabled efficient desktop computing, Ion Torrent semiconductor technology is bringing next generation sequencing to the reach of virtually any lab. In the presentation, principle of semiconductor sequencing will be described and two instruments, Ion Personal Genome Machine and Ion Proton, will be introduced including typical sequencing applications which can be performed on these machines. We will also mention Ion AmpliSeq technology for amplicon sequencing which brings potential for boundless use in research as well in diagnostics.

More information can be obtained on Life Technologies website (www.lifetechnologies.com) after registration to Ion Community.

L-23

NextGENe® complex software tool for next generation sequencing data analysis – introduction and clinical experience

Jan Zastera

Carolina Biosystems, s.r.o., Prague, Czech Republic

Currently many diagnostic laboratories are shifting from Sanger sequencing to higher throughput massively parallel sequencing. A result of the analysis from these systems is the large amount of data which has to be effectively, easily and quickly analyzed. Use of high-quality bioinformatics system except the next generation sequencing instrument itself is then a necessity for such work.

NextGENe® Software is a complete "free standing" analysis package designed for use by bilogists in the analysis of data from Next Generation Sequencing Systems. The icon driven, easy-to-use Windows® interface significantly reduces bioinformatics requirements, provides annotated analysis review, while reducing sequencing errors to improve analysis accuracy and speed. Software is compatible

with all Next Generation Sequencing platforms (Roche, Applied Biosystems or Illumina). Additionally it offers many possibilities to create graphical or tabular reports, filter data and compare the results of analyzes.

Clinical experience with the use of NextGENe® software in the diagnostic context shows that such a comprehensive bioinformatics tool can significantly contribute to a higher sensitivity in detecting mutations in a higher specificity and ultimately reduce the time required to obtain results necessary for the diagnosis.

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