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Noninvasive prenatal testing for congenital heart disease – cell-free nucleic acid and protein biomarkers in maternal blood

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Title: Non-invasive prenatal testing for congenital heart disease – cell-free nucleic acid and protein biomarkers in maternal blood
Short title: Non-invasive biomarkers in fetal heart defects

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Keywords: congenital heart disease, maternal circulation, cell-free nucleic acids, NIPT, biomarkers

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Abstract

Context: Congenital heart disease (CHD) is the most common fetal malformation. Prenatal ultrasonography is routinely applied for the screening of CHD but many factors influence its diagnostic accuracy. The introduction of new biomarkers could facilitate the identification of high-risk pregnancies.

Objective: In our review, our aim was to collect expression studies of cell-free nucleic acids and proteins in maternal circulation. Syndromic CHDs which can be detected by non-invasive prenatal testing (NIPT) techniques were also discussed.

Methods: PubMed and Web of Science databases were screened for studies where the levels of potential CHD biomarkers were measured in maternal blood samples. Available NIPT tests were collected from the providers' resources.

Results: There are nine CHD-associated chromosomal abnormalities, five aneuploidies, and four microdeletions, which are included in NIPT panels.We found eight articles from which five included the analysis of specific cell-free RNA expression and three measurements of protein levels.

Conclusion: Most of the common heart-related chromosomal aberrations can be diagnosed by NIPT. Specific cell-free RNAs and circulating proteins seem to be potential biomarkers for fetal CHDs. The application of these new biomarkers could improve the detection rate at early pregnancy, making it possible to provide optimal perinatal and perioperative management.

Keywords:congenital heart disease, maternal circulation, cell-free nucleic acids, NIPT, biomarkers

Introduction

Congenital heart defects (CHD) are the most common congenital malformations with an incidence of 4-10/1000 live births [1]. Although the pathogenesis of CHD has been extensively studied, the etiology of these malformations is still not clear. CHD remains a serious problem accounting for 30% to 50% of mortality caused by congenital disorders among infants [2] and half of the live birth cases requires one or more surgical procedures in the neonatal period and childhood [3]. In certain types of fetal cardiac anomalies, early prenatal diagnosis may reduce postnatal morbidity and mortality, by providing optimal perinatal and perioperative management [4].Improved outcome was reported for hypoplastic left heart syndrome (HLHS), transposition of the great arteries (TGA) and coarctation of the aorta (CoA) if the prenatal diagnosis had been available [5–7].

Prenatal ultrasonography is the most widely available diagnostic test for fetal CHD but many factors influence its diagnostic accuracy, hence the efficacy of prenatal ultrasound. Unfortunately, detailed fetal echocardiography is limited to a few specialized centers, thus low-risk pregnancies are not routinely screened. The four-chamber view is the gold-standard screening view, on which the bulk of the cardiac lesions manifests alterations, however, other major abnormalities show no sign of disease. Due to the adaptive fetal circulation, one side of the heart can compensate for aberrations present in the other side, which hampers the possibility of detection. Particular cardiac abnormalities present diagnostic difficulties, even in specialized units. If CHD cannot be detected during the ultrasonography examinations, severe comorbidities, such as chromosomal abnormalities, can remain also undiagnosed [8].

Risk factors for CHD include increased nuchal translucency (NT), fetal arrhythmias, major extracardiac anomalies, maternal factors (e.g. metabolic conditions, maternal infections, etc.) and genetic predisposition among others [9]. Nevertheless, the majority of CHD cases occurs in the low-risk population, which emphasizes the importance of identifying factors that assign pregnancy to the high-risk group of CHD [10]. In our review, our aim was to collect and discuss expression studies of cell-free nucleic acids and proteins in maternal circulation, which reveal new opportunities for CHD research and diagnosis.The introduction of new biomarkers could facilitate the identification of high-risk pregnancies, thereby enabling a better diagnostic efficacy.

Clinical significance

Prenatal ultrasonography is routinely applied for the screening of fetal CHD but many factors influence its diagnostic accuracy. Detailed fetal echocardiography is limited to a few specialized centers, thus low-risk pregnancies are not routinely screened. The introduction of new biomarkers could facilitate the identification of high-risk pregnancies, thereby enabling a better diagnostic efficacy of CHD. Expression studies of cell-free nucleic acids and proteins in the maternal circulation reveal new opportunities for CHD research and diagnosis. The application of these new biomarkers could improve the detection rate at early pregnancy, making it possible to provide optimal perinatal and perioperative management.

1.

Circulating cell-free nucleic acids (cfNA)

cfNAs can be found in the bloodstream, where they circulate freely, bound to lipoprotein molecules or encapsulated within extracellular vesicles [11]. These DNA and different kind of RNA molecules are thought to originate from apoptotic and necrotic cells that release their contents into the circulation during their degradation [12]. cfNAs offers a non-invasive approach to the early diagnosis of a wide range of clinical disorders.

The detection of circulating cfNAs in maternal blood has opened the door for non-invasive prenatal screening, avoiding the necessity of risk-associated invasive procedures including amniocentesis and chorionic villus sampling. Fetal-specific cfNAsare originated primarily from the trophoblast layer of the placenta, which is in direct contact with the maternal circulation. Placenta-derived cfNAscan be detected in the bloodstream from the 7thweek of pregnancy with levelsincreasing as the gestation progresses and vanish quickly after birth[13]. Circulating cell-free fetal DNA adds up toroughly 3–13% of the total cell-free maternal DNA during pregnancy. Different types of placenta-specific RNAs also present in the maternal blood, including mRNA, microRNA (miRNA) and long noncoding RNA (lncRNA) species.As free RNA molecules are prone to degradation in theextracellular environment, it is believed that some type ofcirculating RNAs is protected from nuclease activity by packaging into extracellular vesicles, making complexes with lipoproteins or bounding to protein molecules[14].

Circulating cell-free DNA

Approximately 25 to 40% of CHD cases are related to genetic syndromes or associated with other birth defects [15]. Some of the syndromic CHDs can be detected by NIPT techniques, which enable high-throughput screening using next-generation sequencing (Table 1.)[16,17]. It

is based on the analysis of circulating fetal-specific cell-free DNA in the maternal circulation to screen for common chromosome conditions. Several NIPT companies offer very sensitive and specific screening tests for aneuploidies, including trisomy 21, 13, 18, and sex aneuploidies. CHD accompanies approximately 40-50% of trisomy 21 cases (Down syndrome) and is a determining factor of survival [18]. Fetuses with trisomy 18 (Edward syndrome) or trisomy 13 (Patau syndrome) have a low rate of survival, and the majority of fetuses die before birth. In a registry-based study performed in Europe, about 76–83% of live-born babies with trisomy 18, and 51-64% of trisomy 13 cases 51–64% was reported to have CHD [19]. Turner syndrome also called 45,X monosomy is associated with heart development abnormalities in 25-45% of live births and more frequent if perinatal death cases are taken into account. In patients with Klinefelter syndrome (47,XXY), there is a 50% incidence of CHD.

Congenital heart anomalies also occur in partial aberration syndromes, from which a few are already included in NIPT companies' screening panels: DiGeorge, 1p36, Wolf-Hirschorn, and Jacobsen syndromes (Table 1.). DiGeorge syndrome, also known as 22q11.2 deletion syndrome is considered as the most common human microaberration.

Circulating cell-free mRNA

Apart from the well-known cell-free fetal DNA, different types of placenta-specific RNAs could be applied as non-invasive markers for CHD (Table 2.). The RNA profile of chorionic villous samples and intraembryonic mesoderm derivates, including heart and NT, are shown to beanalogous during the first trimester of pregnancy [20]. Arcelli et al. hypothesized that aberrant expression of placental protein-coding genes related to cardiogenesis can be measured in the maternal circulation [21]. In the first stage of their study, they screenedplacental mRNA profile of women bearing fetus with CHD and healthy pregnant controls. They found that the levels of *MAP4*, *MYL7*, *P4HA2*, *PAPP-A*, *SAV1*, *TNXB*, and *TXN* were altered in the patient group.In the second stage of the study, aberrant mRNA expression was validatedin 2nd-trimester maternal plasma samples for *SAV1*, *TXNB*, *PAPP-A*, *TXN*, and *MYL7*. The calculated the sensitivities were 95%, 94%, 79%, 45%, and 42% at 10% false positive rate (FPR), respectively.Both the developmental and validation modelCHD groupsincluded different kinds of lesions. In another study from the same group, Nanostring technology was applied for the identification ofaberrant gene expression in 2nd-trimester maternal plasma samples, and top findings were subsequently measured by qPCR.The pool of six differently expressed genes including *FALZ*, *PAPP-A*, *PRKACB*, *SAV1*, *STK4*, and *TNXB2* was found to have a 66.7% detection rate at 10% FPR for CHD [22].

Circulating cell-free microRNA

Data on the use of circulating microRNAs (miRNAs) as biomarkers of several diseases are emerging. miRNAs are evolutionary conserved, small (20-26 nucleotides), non-coding

RNA molecules that play an important role in the regulation of eukaryotic gene expression. miRNAs participate in the fine-tuning of several fundamental biological processes. miRNA expression profile changes during heart development [23] as well as the progression of heart-related conditions, indicating that aberrant miRNA profile is involved in cardiovascular diseases [24]. The possibility of using miRNAs to investigate the consequences of CHDs in children and adults has also recently become an intensively researched field [25].

Cell-free miRNAs are shown to be quite stable in the circulation and they are also resistant to adverse physiological circumstances such as multiple freezing/thawing cycles, and extended storage[26].Expression studies of heart development related miRNAs in maternal circulation reveal new opportunities for CHD research and diagnosis.Consequently, it can be postulated that these specific miRNAs of placental origin could be applied as potential biomarkers for fetalCHD[27].Zhu et al. performed SOLiD sequencing to compare the miRNA profile of pooled serum samples from women carrying afetus with CHD and healthy pregnant women.All the affected cases were VSD, ASD, orToF. Following qPCR validation assays, they found four miRNAs, namely*miR-19b*, *miR-22*, *miR-29c*, and *miR-375*, which were significantly upregulated in the patient groupwith an area under the receiver operating characteristic curve (AUC)of 0.79, 0.671, 0.767 and 0.693, respectively.[28].Kehler et al found elevated *hsa-miR-99a*level as a possible biomarker for the detection of CHD by analyzing maternal plasmasamples[29].The *miR-99a/let7c* miRNA cluster is located in the chromosome region 21q21.1 and has been shown to control cardiomyogenesis in embryonic stem cells[30].Lázár et al. has also analyzed *let-7c* expression in the maternal circulation and found that similarly to *hsa-miR-99a*, it is also overexpressedin cases of fetal cardiac malformations [31].

Circulating cell-free long noncoding RNA

Long noncoding RNAs (lncRNA) are more than 200 nucleotides long, poorly conserved, and the majority of them is species and tissue-specific. Possible mechanisms by which lncRNAs can alter gene expression include mRNA decay, mRNA stabilization, or miRNA sponge. lcnRNAs play a major role in the regulation of organ development, organ physiology, and pathophysiology. In the fetal heart, lncRNAs are associated with genes involved in development and programming [32]. They are known to be differentially activated at the fetal and postnatal stages of heart development [33]. However, no individual human lncRNA has been causally associated with CHD until now. Nevertheless, a recent study compared the expression profiles of cardiac tissue from fetuses with VSD, one of the most common CHDs, and normal hearts [34]. More than 1500 lncRNAs were differentially expressed in hearts with VSD at the gestational age between 17 and 20 weeks.

CHD associated lncRNAs were detected in the placenta, therefore circulating lncRNAs may be utilized as early non-invasive biomarkers of CHD, similarly to other types of RNAs. To explore the clinical utility of lncRNAs as biomarkers to predict fetal CHD in pregnant women,

Gu et al screened the lncRNA profile by microarray technology, and the significant findings were further investigated by Gene Ontology, pathway and network analysis. The disease group included only VSD, ASD, and ToF samples. The differential expression of the top lncRNAs was validated by qPCR and ROC curves were calculated.They found that *ENST00000436681* and *ENST00000422826* were upregulated, while *AA584040*, *AA709223*, and *BX478947* were downregulated in plasma samples of the affected group, with AUC values calculated as 0.892, 0.817, 0.755, 0.882, and 0.886, respectively [35].

Circulating protein molecules

Circulating protein molecules that have been applied in the prenatal setting for a long time, are also offers a possibility for CHD screening. Llurba et al suggest that in case of CHD, an imbalance of angiogenic-antiangiogenic factors present in both the maternal and fetal circulation. In their first study, they proposed PIGF as a first-trimester biomarker for the screening of conotruncal and valvular defects [36]. In the second study, angiogenic factors and hypoxia markers were evaluated in fetal heart, umbilical cord and maternal blood samples collected in the second and third semesters [37]. *VEGF-A*, s-Flt1, HIF-2α, *HO-1*, *SOD-1* mRNA expression was significantly elevated in affected fetal heart samples compared to controls, and surprisingly no difference was measured in HIF-1α and PlGF. In the circulation of women carrying CHD fetus, PlGF levels were significantly lower and sFlt-1 levels were significantly higher. Curti et al performed a similar study on 3rd-trimester plasma samples using the Alere PlGF Test, and they found that in line with the previous results, the PlGF concentration measured in the affected group was significantly lower than in the control samples [38].

Chen et al. implementedserum proteomics analysis for exploringcirculating biomarkers of fetal CHD in maternal blood[39].In the discovery phase, they performedshotgun quantitative proteomics applying iTRAQ(isobaric Tagging for Relative and Absolute Quantification)approach for comparing protein profiles of pooled serum samples in the affected and control groups.The top findings were validated with MRM-MS and were further evaluated in a large independent cohort using ELISAmethod. They identified a biomarker panel consisting of cytoskeleton pathway proteins (LMNA, FLNA, TPM4, and ACTG1)all of which were significantly lower in CHD maternal serum comparing to healthy controls. The combination of the four candidates resulted in an AUC of 0.938 (95% CI, 0.905-0.970), and the sensitivity and specificity were 95.0% (95% CI, 86.1%- 99.0%) and 83.9% (95% CI, 76.0%- 90.0%), respectively.Interestingly, theyfound that serum level of LMNA was very low in nonpregnant women and increased with gestation age in pregnant women, suggesting a specific role inpregnancy.

Discussion

Fetal-specific cfNAs are originated from the placenta and can be found in the maternal circulation during pregnancy. Apart from the well-known cfDNA, different types of placenta-specific RNAs also present in the maternal blood, including mRNA, miRNA, and lncRNA. Farina and Arcelli suggest that the analysis of the placenta-derived RNA species could be applied as a non-invasive screening tool of CHD [40]. Placentally expressed genes related to cardiogenesis can be detected in the maternal circulation, which may reflect the development of the birth defect, hence gives an opportunity for investigation during early pregnancy.

Comparative proteomic analysis has been widely used in discovering new biomarkers for various pregnancy complications and fetal abnormalities. Dysregulation of these proteins not only opens the door for the development of new non-invasive biomarkers for CHDs but also reflects the molecular origins of the disease. Disturbed angiogenesis has been implicated in abnormal heart development and placentation. Both pregnant women bearing fetuses with CHD and women affected by preeclampsia (PE) often show angiogenic imbalances. Angiogenic factors including s-Flt1 and PlGF were found to be dysregulated in CHD and what is more, these proteins are routinely used in the first-trimester screening of PE. Boyd et al. conducted a register-based cohort study and they found that linked pathophysiological mechanisms may be implicated in some CHDs and preterm PE [41]. Based on the strong associations across pregnancies they suppose that PE predisposes to the development of CHDs. Cytoskeletal proteins are essential for the structure and function of the cardiac myocytes, and some of them are probably pregnancy-specific. The study of Chen et al. indicated that dysregulation of these proteins might reflect disturbance of cardiogenesis and could be applied in the screening of CHD pregnancies. However, further studies are needed to understand the exact mechanism for the downregulation of cytoskeletal proteins in heart anomalies. The advantages and limitations of the application of different cf-NA and protein biomarkers as NIPT tools are summarized in Table 3.

Conclusion

In spite of that CHDs are the most common group of birth defects, current prenatal screening is not able to detect high-risk cases effectively. Expression studies of cfNAs and proteins in the maternal circulation reveal new opportunities for CHD research and diagnosis. Most of the common heart-related chromosomal aberrations can be diagnosed using NIPT methodology by the analysis of cell-free fetal DNA. Based on our review, cell-free RNAs and circulating proteins seems to be potential biomarkers for fetalCHDs and could be used to identify high-risk pregnancies to be addressed for fetal echocardiography.The application of these new biomarkerscould improve the detection rate at early pregnancy, making it possible to provide optimal perinatal and perioperative management.

Disclosure of interest

The authors report no conflicts of interest.

Abbreviations

ASD: Atrial Septal Defect
AS: Aorta Stenosis
AUC: Area Under the receiver operating characteristic Curve
AVSD: Atrioventricular Septal Defect
BAV: Bicuspid Aortic Valve
cfNA: cell-free Nucleic Acid
CHD: Congenital Heart Disease / Congenital Heart Defect
CoA: Coarctation of the Aorta
DORV: Double Outlet Right Ventricle
ELISA: Enzyme-Linked Immunosorbent Assay
FPR: False Positive Rate
HLHS: Hypoplastic Left Heart Syndrome
IAA: Interrupted Aortic Arch
iTRAQ: isobaric Tagging for Relative and Absolute Quantification
lncRNA: long noncoding RNA
mRNA: messenger RNA
miRNA: microRNA
MRM-MS: Multiple Reaction Monitoring Mass Spectrometry
MS: Mitral Stenosis
NIPT: Non-Invasive Prenatal Testing
NT: Nuchal Translucency
PDA: Patent Ductus Arteriosus
PS: Pulmonary Stenosis
ROC: Receiver Operating Characteristic
SOLiD: Sequencing by Oligonucleotide Ligation and Detection
TGA: Transposition of the Great Arteries
ToF: Tetralogy Of Fallot
VSD: Ventricular Septal Defect

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Syndrome	CHD frequency[1]	CHD type[2]	NIPT panel[3-8]
Aneuploidies			
Down syndrome (Trisomy 21)	40-50%	ASD, VSD, AVSD, ToF	all
Edwards syndrome (Trisomy 18)	90-100%	ASD, VSD, PDA, TOF, DORV, CoA, BAV	all
Patau syndrome (Trisomy 13)	80%	ASD, VSD, PDA, HLHS	all
Turner syndrome (45,X0)	25-35%	CoA, BAV, AS, HLHS	all
Klinefelter syndrome (47,XXY)	50%	PDA, ASD	all
Microdeletions			
DiGeorge syndrome (22q11.2 deletion)	75%	ToF, IAA-B, VSD, truncus arteriosus, DORV	BGI, Sequenom, Natera, Illumina, Roche, Lifecodexx
Jacobsen syndrome (11q23 deletion)	56%	VSD, ASD, TA, DORV, BAV, AS, HLHS, MS, CoA	Sequenom
Wolf-Hirschhorn syndrome (4p16.3 deletion)	50%	ASD, PS, ToF, VSD, PDA	BGI, Sequenom, Illumina
1p36 syndrome	35%	Septal defects, PDA, valvular abnormalities, CoA, ToF, cardiomyopathy	BGI, Sequenom, Natera, Illumina

Table 1. Screening of chromosomal abnormality-relatd syndromic CHDs and available NIPTpanels.

[1][15]
[2][42]
[3][43]
[4][44]
[5][45]
[6][46]
[7][47]
[8][48]

Gestation	Blood component	Type of biomarker	Potential biomarkers	Diagnostic measure	Methods	Ref.
				Sensitivity		
20–22 weeks	Whole blood (EDTA)	mRNA	SAV1 ↑ TNXB ↑ PAPP-A ↑ TXN ↑ MYL7 ↑	95% 94% 79% 45% 42%	RT-PCR*	[21]
19–24 weeks	Plasma	mRNA	PAPP-A ↑ PRKACB ↑ SAV1 ↑ STK4 ↑ TNXB2 ↑	18% 18% 28% 41% 33%	Nanostring RT-PCR*	[22]
11-13 weeks	Plasma	protein	PLGF ↓	-	ELISA*	[36]
2nd-3rd trimester	Plasma	protein	s-Flt1 ↑ PLGF ↓	-	ELISA*	[37]
				AUC		
22-26 weeks	Serum	protein	LMNA ↓ FLNA ↓ TPM4 ↓ ACTG1 ↓	0.867 0.766 0.760 0.760	iTRAQ MRM-MS ELISA*	[39]
18-22 weeks	Serum	miRNA	*miR-19b* ↑ *miR-22* ↑ *miR-29c* ↑ *miR-375* ↑	0.79 0.671 0.767 0.693	SOLiD sequencing RT-PCR*	[28]
2nd-3rd trimester	Plasma	miRNA	*miR-99a* ↑	-	RT-PCR*	[39]
22-26 weeks	Plasma	lncRNA	*ENST00000436681*↑ *ENST00000422826*↑ *AA584040* ↓ *AA709223* ↓ *BX478947* ↓	0.892 0.817 0.755 0.882 0.886	Microarray RT-PCR*	[35]

Table 2. Potential cell-free RNA and protein biomarkers for CHD in the maternal circulation. ↑: overexpression, ↓: underexpression. *Applicable diagnostic tool

Biomarkers	Advantages	Disadvantages
cf-DNA	Standardized High sensitivity Translated into the clinic	Applicable only for specific CHD-related chromosomal aberrations
cf-mRNA	Reflect the status of intracellular processes	Degradation, instability, low abundance Detection difficulties Low reproducibility
cf-miRNA	Stable Easily detectable Tissue-specific expression Extensively researched	Lacks validation, standardization, and internal controls Influencing factors (e.g. hemolysis) Large sample-to-sample variability
cf-lncRNA	Stable Easily detectable Tissue-specific expression	Lacks validation, standardization, and internal controls Large sample-to-sample variability
Protein	Easily detectable Cost-efficient	Usually not tissue-specific, can reflect the disease state of any organ Poor signal-to-noise ratio

Table 3. Advantages and disadvantages of circulating cf-NA and protein biomarkers in NIPT