A new method for non-invasive prenatal diagnosis of Down syndrome using MeDIP real time qPCR

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ABSTRACT

During the last decade, the area of non-invasive prenatal diagnosis (NIPD) has rapidly evolved. Several methodological approaches have been presented and demonstrated a proof of concept for the NIPD of chromosomal aneuploidies. The two most promising methods are NIPD using next generation sequencing technologies and NIPD using Methylation DNA Immunoprecipitation (MeDIP) with real time qPCR. Both approaches have been validated with blind studies and have >99% accuracy. NIPD using next generation sequencing is achieved by high throughput shotgun sequencing of DNA from plasma of maternal women followed by ratio comparisons of each chromosome sequence tag density over the median tag density of all autosomes (z-score analysis). The MeDIP real time qPCR method, which is described in this review in more detail, is based on the identification of differentially methylated regions (DMRs) and their use in discriminating normal from abnormal cases. More than 10,000 DMRs were identified for chromosomes 13, 18, 21, X and Y using high resolution oligo-arrays that can be potentially used for the NIPD of aneuploidies for chromosomes 13, 18, 21, X and Y. Both NIPD methods have several advantages and limitations and it is believed that they will soon be implemented in clinical practice. With the continuous advancements of genetic methodologies and technologies, we predict that within the next 10 years we will be able to provide NIPD for all common and rare genetic disorders where the molecular basis is known.

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1. Introduction

Non-invasive prenatal diagnosis (NIPD) is one of the most fascinating research areas in molecular medicine of the last decade. The

medical significance of the development of NIPD is of great importance as it can potentially be offered to all pregnancies, presents no risk of loss of the pregnancy and provides a more effective prenatal diagnosis compared to currently used invasive methods.

The identification and isolation of fetal DNA by non-invasive means, which will then be analyzed using molecular methodologies, are the key issue toward NIPD. The first evidence for circulating nucleic acids in the peripheral blood was shown by Mandel and Métals (1948). The isolation in 1997 of fetal cells from maternal blood (Bianchi et al., 1997) and the identification of small amounts of cell free fetal DNA in maternal plasma (cffDNA) (Lo et al., 1997) have greatly facilitated the

development of NIPD. However, the limited amounts of cffDNA (3–6%) (Lo et al., 1998), as well as the fact that it is 50% identical and co-exists with maternal DNA, poses major challenges toward the development of NIPD.

2. Toward NIPD for genetic disorders

NIPD was initially attempted using the strategy of the isolation of intact fetal cells from maternal blood using fluorescence in situ hybridization (FISH) and other molecular analyses (Hassold et al., 1996). However, the very small number of fetal cells (1 in 350,000 maternal cells) (Hamada et al., 1993) and the isolation of these cells, present major difficulties toward achieving this goal. Alternatively, a more feasible approach for the development of NIPD was to use the relatively abundant cffDNA present in maternal circulation. Initially NIPD was achieved for genetic loci of absolute fetal specificity, such as Y-chromosome specific markers (Lo et al., 1998) and the fetal RhD gene (Faas et al., 1998). Therefore, the development of NIPD for the diagnosis of X-linked disorders (Anon, 2007) and rhesus D positive fetuses (Wright and Burton, 2009) was relatively straightforward and very accurate. However, the development of NIPD for genetic loci of non-absolute fetal specificity, such as autosomal fetal DNA aneuploidies for chromosomes 13, 18, 21, X and Y proved much more challenging (Chim et al., 2005; Tsui et al., 2010; Tong et al., 2010a). This has mainly been achieved using sodium bisulphate conversion and/or methylation sensitive restriction enzymes with conventional, as well as digital PCR methods (Lo et al., 2007a; Fan and Quake, 2007).

The most promising demonstrations of NIPD were based on the use of differentially methylated markers (DMRs), which have differences in their methylation status between fetal DNA and maternal DNA (Chim et al., 2005; Tsui et al., 2010; Tong et al., 2010a; Poon et al., 2002; Old et al., 2007; Lo et al., 2007b; Chim et al., 2008). Several studies used DMRs in combination with informative SNPs and methylation sensitive restriction enzymes and/or sodium bisulphate conversion methods (Tsui et al., 2010; Tong et al., 2010a,b). These methods were compromised by the limited number of known DMRs, and the even more limited number of informative SNPs or methylation sensitive sites in these regions. In addition, complete sodium bisulphate conversion is rarely achieved and degradation of DNA always occurs after sodium bisulphate treatment. These limitations compromised the accuracy and thus the diagnostic usefulness of these NIPD methods. To overcome the above limitations, our team aimed in identifying a very high number of DMRs of high quality, as well as in developing a new NIPD approach for genetic disorders (Papageorgiou et al., 2009, 2011).

3. NIPD of Down syndrome using MeDIP and real time qPCR

The identification of new DMRs on chromosomes 13, 18, 21, X and Y was achieved by using a method consisting of methylated DNA immunoprecipitation with high resolution oligo-arrays (MeDiP on a Chip) (Papageorgiou et al., 2009; Rakyan et al., 2008), and is shown in Fig. 1. In principle, DNA from non-pregnant female blood and 1st and 3rd trimester placentas were immunoprecipitated in two separate experiments, using a specific antibody for CG-methyl sites. The isolated hypermethylated regions (MeDiP DNA) and non-hypermethylated regions (input DNA) of female blood and placenta samples were co-hybridized on a high resolution oligo-array. The microarray platform was a high resolution oligo-array of chromosomes 13, 18, 21, X and Y. Differentially methylated regions were identified using the SW-ARRAY algorithm previously used for copy number variation (CNV) calling, as described by Price et al. (2005). As a result, more than 10,000 candidate DMRs were isolated (Papageorgiou et al., 2009). This study provided numerous DMRs that could be potentially used for NIPD of aneuploidies of chromosomes 13, 18, 21, X and Y.

Among the ~2,000 DMRs identified on chromosome 21, the most suitable 12 DMRs of chromosome 21 were selected based on the following three criteria: firstly, the region should be hypermethylated in the placenta and hypomethylated in the maternal peripheral blood; secondly, the methylation status should be the same in the first and third trimester placentas; and thirdly the methylation level should be above a certain threshold value determined from microarray analysis. The methylation levels of all 12 of these selected DMRs on chromosome 21 were re-confirmed by real time qPCR using peripheral blood of non-pregnant women as well as 1st and 3rd trimester placentas from individuals not screened in the initial microarray experiments. This work verified that these 12 DMRs consistently showed hypomethylation in peripheral blood and hypermethylation in placentas (Papageorgiou et al., 2009) and also showed that the degree of methylation varied between individuals, an observation which is called interindividual methylation.

In order to demonstrate that these DMRs can be used to identify fetal DNA in maternal peripheral blood and discriminate normal from Down syndrome cases, several samples of peripheral blood from normal as well as trisomy 21 pregnancies were tested and analyzed. Fig. 2 demonstrates the ratio value calculation of a Down syndrome or a normal case compared to the median value of normal control cases. These results, and statistical analysis of the ratio value differences between normal and abnormal cases, permitted the development of a new NIPD for trisomy 21 which is based on measuring and comparing fetal specific methylation ratios between unknown and known normal pregnancies (Fig. 2).

This newly developed NIPD method for trisomy 21 using MeDIP and real time qPCR was initially tested in 40 maternal peripheral bloods, of which 20 were from normal and 20 from Down syndrome pregnancies. A combination of results obtained from 8 out of 12 DMRs tested led to the correct diagnosis of all 20 normal and 20 Down syndrome pregnancies tested (Papageorgiou et al., 2011). The method involves MeDIP of known normal and unknown DNAs, freshly extracted from peripheral blood at 11–13 weeks of gestation, real time qPCR of eight DMRs and statistical analysis using a diagnostic equation (Papageorgiou et al., 2011) (Fig. 2). The new NIPD method was further validated in a blind study with 40 additional maternal peripheral blood samples, 26 of which were women bearing a normal fetus and 14 bearing a Down syndrome fetus. Prediction values obtained from normal and trisomy 21 cases demonstrated the correct diagnosis of all samples, providing 100% specificity and 100% sensitivity (Papageorgiou et al., 2011).

4. NIPD in clinical practice

Currently, two methods have been developed and validated, and have demonstrated a near 100% accuracy. One method is based on next generation sequencing (Fan et al., 2008; Chiu et al., 2008), and the other one is based on MeDIP and real time qPCR (Papageorgiou et al., 2009, 2011). NIPD by next generation sequencing is achieved by high throughput shotgun sequencing of DNA from plasma of maternal peripheral blood, followed by ratio analysis of each chromosome sequence tag density over the median tag density of all autosomes using a z-score analysis (Fan et al., 2008). The method was further validated using 753 plasma samples of which 86 were from Down syndrome pregnancies and provided 100% and 97.9%, sensitivity and specificity, respectively (Chiu et al., 2011). Recently, an additional validation study using next generation sequencing was carried out in 1696 cases and demonstrated 98.6% and 99.8%, sensitivity and specificity, respectively (Palomaki et al., 2011). The MeDIP and real time qPCR based approach (Papageorgiou et al., 2009, 2011), was developed and validated using 80 samples of maternal peripheral blood, from 46 women bearing a normal fetus and 34 bearing a Down syndrome fetus, and provided 100% specificity and 100% sensitivity (Papageorgiou et al., 2011). Recently, a larger cohort
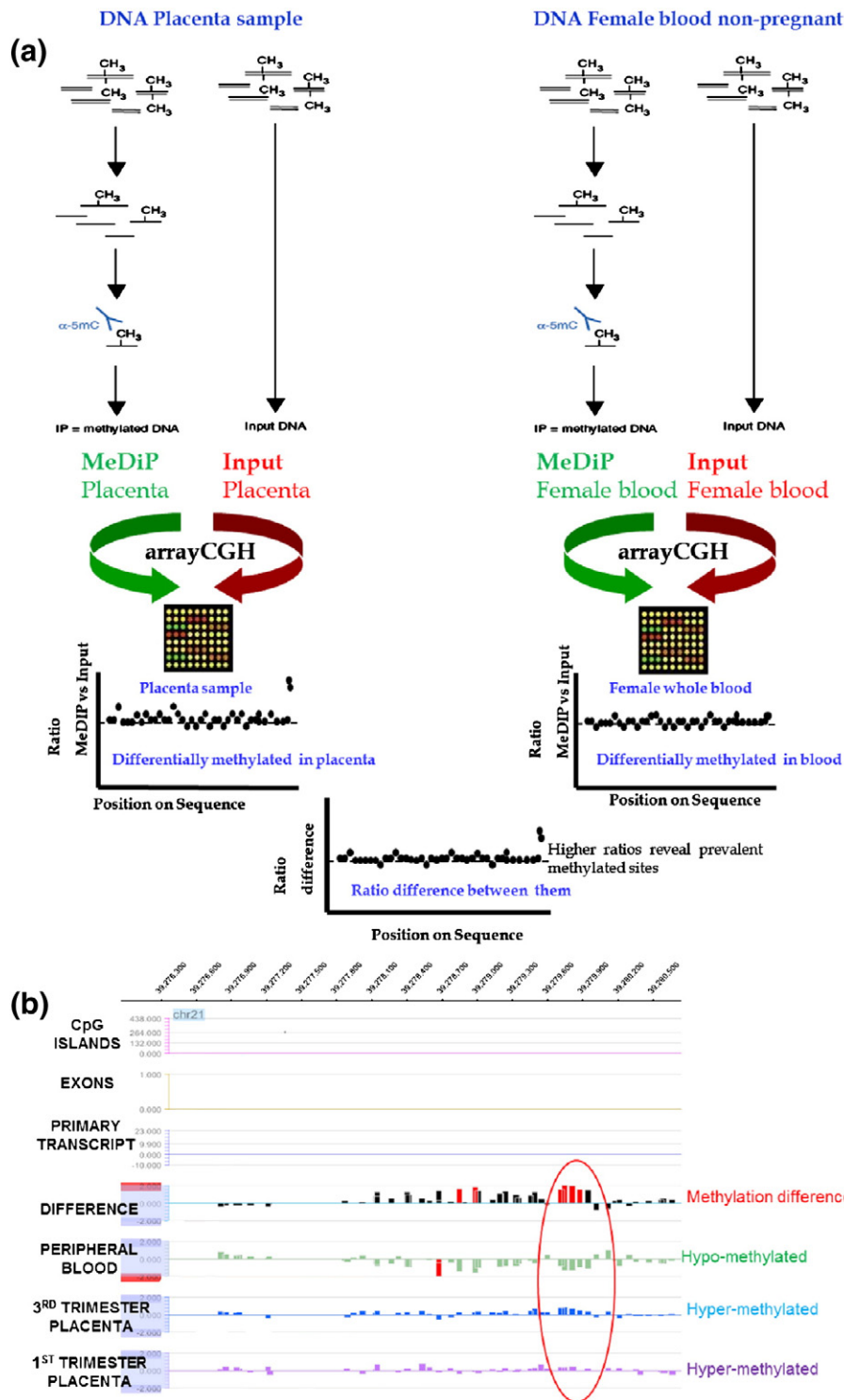
Fig. 1. Identification of DMRs using MeDIP on a Chip. (a) Extracted DNA placenta sample and DNA female blood non-pregnant sample were immunoprecipitated (MeDIP) using an antibody targeting 5-methylcytocines of CG dinucleotides. An aliquot of unprocessed DNA (Input DNA) was not immunoprecipitated. MeDIP DNA and Input DNA were then co-hybridized on high resolution oligo-arrays specific for chromosomes 13, 18, 21, X and Y with median probe spacing of 225 bp, 170 bp, 70 bp, 340 bp and 20 bp, respectively. Differentially methylated regions (DMRs) in non-pregnant female blood and placental DNAs were identified and ratio differences between them were calculated. (b) The plot shows the methylation levels in a region of chromosome 21. The red circle shows an informative DMR region because it is hypomethylated in DNA from peripheral blood of non-pregnant women and hypermethylated in DNA from 1st and 3rd trimester placentas. The methylation difference between peripheral blood and placenta is significant.

of 175 samples was tested for further validation and demonstrated the same level of accuracy (Tsaliki et al., submitted for publication). However, prior to considering the method ready for implementation in clinical practice, an even larger validation study consisting of 1000 samples will soon be initiated.

Comparison between the two methods shows several similarities. They are both considered accurate, with sensitivity and specificity close to 99% (Verweij et al., 2012). Both methods can be offered as early as the 10th week of gestation and can yield rapid results, with a higher turn-over time of 15–21 days for NIPD using next generation
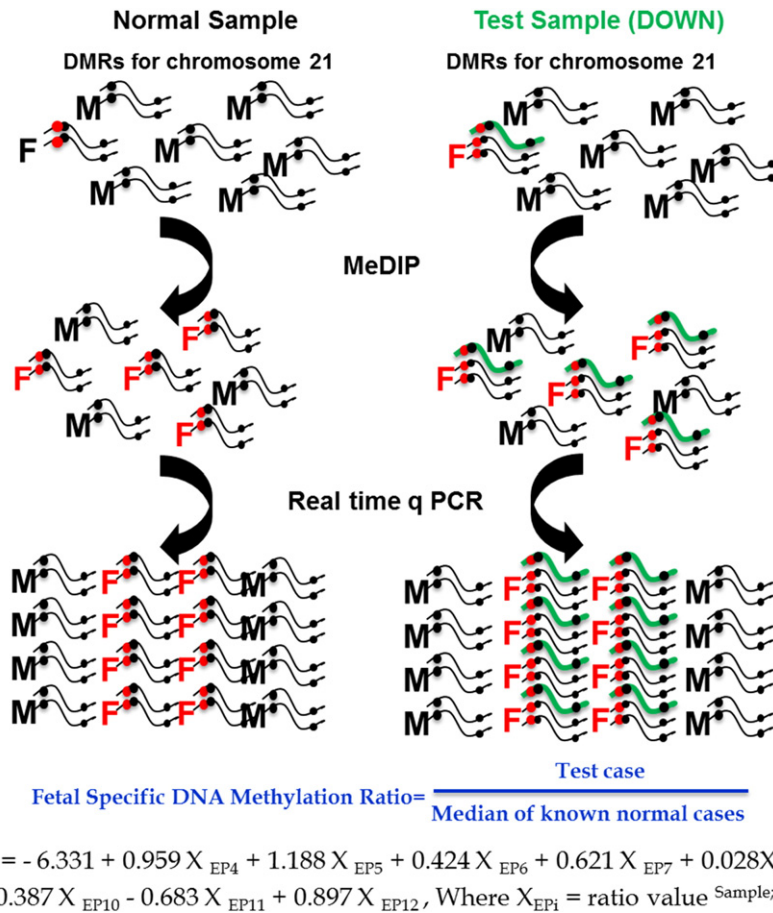
$$D = -6.331 + 0.959\,X_{EP4} + 1.188\,X_{EP5} + 0.424\,X_{EP6} + 0.621\,X_{EP7} + 0.028 X_{EP8}$$
$$+ 0.387\,X_{EP10} - 0.683\,X_{EP11} + 0.897\,X_{EP12}, \text{ Where } X_{EPi} = \text{ratio value}^{Sample;\,EPi}$$

Fig. 2. Fetal specific DNA methylation ration permits NIPD of Down syndrome. DNA from peripheral blood of known normal and unknown pregnancies are first immunoprecipitated using the MeDIP protocol to enrich fetal DNA in regions (DMRs) that are known to be hypermethylated in the fetus and hypomethylated in the mother. Real time qPCR is used to quantify all DMRs on chromosome 21 and measures the fetal specific DNA methylation ratio between the test sample (Down syndrome in this figure) and a median of known normal samples. A D value is derived using the ratios from several DMRs and provides the final result of the test sample (Down syndrome in this figure). F: fetal, M: maternal, red dots: hypermethylated DNA, and black dots: hypomethylated DNA.

sequencing compared to a lower turn-over time of 2–3 days for NIPD using MeDIP and real time qPCR. As stated earlier, the NIPD method using next generation sequencing has been validated using a larger number of samples and thus, has been more extensively proven to be accurate and robust and is therefore expected to be implemented in clinical practice first. The NIPD method using MeDIP and real time qPCR will soon undergo additional validation with a cohort of 1000 samples, which is necessary prior to clinical implementation. Such large validation studies will further improve and refine the statistical analysis and, more importantly, identify additional parameters that may influence the results that lead to false positive or false negative misdiagnosis. In regard to their cost and complexity, NIPD using next generation sequencing is currently more expensive (~1900 USD) and more complex as it requires specialized or complex laboratory equipment, software, infrastructure or know-how, while NIPD using MeDIP and real time qPCR is considerably less expensive (~400 Euros) and is less complex as it uses standard molecular biology techniques and infrastructure.

5. NIPD of genetic disorders in the years to come

Since the 1970s, prenatal diagnosis has been offered through the invasive procedures of CVS, amniocentesis and chordocentesis using chromosomal analysis, FISH and DNA methods (Fig. 3). With the rapid advances in technologies and methodologies, the scientific community has managed to increase the resolution of genome analysis. Such achievements permitted the detection of DNA defects from

the size of megabases to single bases. These advances led from the karyotype and the detection of aneuploidies, to FISH and the identification of common microdeletion and subtelomeric rearrangements, to array CGH and identification of causative copy number changes, and currently to DNA molecular analyses and the identification of causative point mutations. It took us 50 years to move from the use of invasive prenatal diagnosis for chromosome analysis to the very high resolution genomic analyses and consequently this has led to the provision of prenatal diagnosis of an increasingly greater number of genetic disorders, as well as in significant improvements in accuracy (i.e. "more and better") (Fig. 3).

For the last 20 years to make NIPD a reality has been the goal of several groups (Fig. 3). It appears that finally, now in 2012, a new era of non-invasive prenatal diagnosis has begun. Before the end of this year we will be ready to provide NIPD of Down syndrome and very soon after, NIPD for other aneuploidies. The rapid advancements in methodologies such as MeDIP and real time qPCR, next generation sequencing, digital PCR, array-CGH and other emerging technologies will permit within the next 10 years, provision of NIPD for all other common and rare genetic disorders. Therefore, the continuous development and improvement of NIPD methodologies will not only provide more and highly accurate prenatal diagnosis of genetic disorders ("more and better"), but will do so without any risk for the fetus. It will therefore be a more effective approach for prenatal diagnosis which may be offered to all pregnant women regardless of age or indication.(i.e. "safe, effective and holistic") (Fig. 3). By the year 2020, NIPD will be a reality for the testing of the most common and rare genetic disorders.
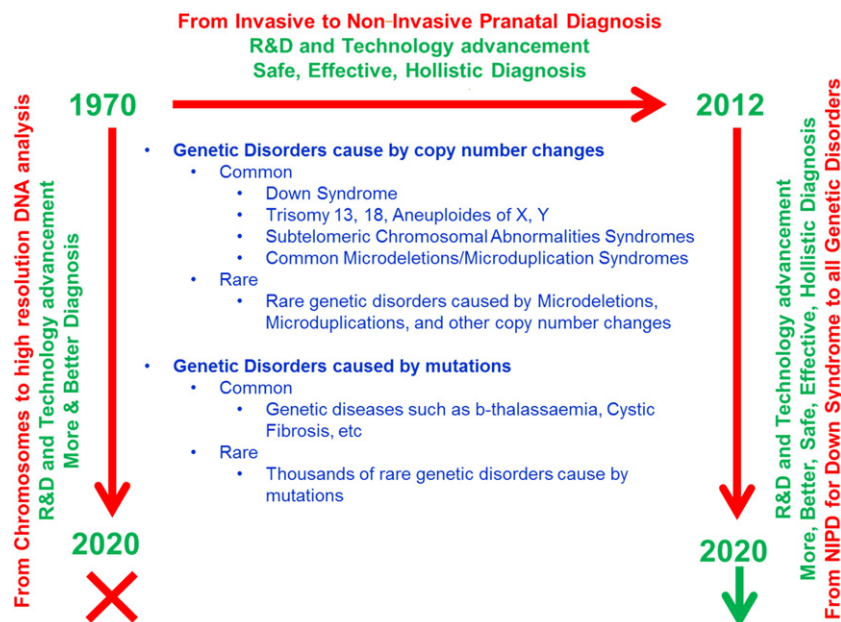
Fig. 3. From chromosomes to high resolution DNA analysis and from invasive to non-invasive prenatal diagnosis.

Conflict of interest

Philippos C. Patsalis declares a conflict of interest as he has filed with others a PCT patent application for the NIPD/MeDIP and real time qPCR based approach (PCT Patent Application No. PCT/1B2011/000217).

References

ACOG Committee opinion no. 360: sex selection. Obstetrics and Gynecology 109, 475–478.

Bianchi, D.W., Williams, J.M., Sullivan, L.M., Hanson, F.W., Klinger, K.W., Shuber, A.P., 1997. PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. American Journal of Human Genetics 61, 822–829.

Chim, S.S., Tong, Y.K., Chiu, R.W., Lau, T.K., Leung, T.N., Chan, L.Y., Oudejans, C.B., Ding, C., Lo, Y.M., 2005. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. Proceedings of the National Academy of Sciences of the United States of America 102, 14753–14758.

Chim, S.S., Jin, S., Lee, T.Y., Lun, F.M., Lee, W.S., Chan, L.Y., Jin, Y., Yang, N., Tong, Y.K., Leung, T.Y., Lau, T.K., Ding, C., Chiu, R.W., Lo, Y.M., 2008. Systematic search for placental DNA-methylation markers on chromosome 21: toward a maternal plasma-based epigenetic test for fetal trisomy 21. Clinical Chemistry 54, 500–511.

Chiu, R.W., Chan, K.C., Gao, Y., Lau, V.Y., Zheng, W., Leung, T.Y., Foo, C.H., Xie, B., Tsui, N.B., Lun, F.M., Zee, B.C., Lau, T.K., Cantor, C.R., Lo, Y.M., 2008. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. Proceedings of the National Academy of Sciences of the United States of America 105, 20458–20463.

Chiu, R.W., Akolekar, R., Zheng, Y.W., Leung, T.Y., Sun, H., Chan, K.C., Lun, F.M., Go, A.T., Lau, E.T., To, W.W., Leung, W.C., Tang, R.Y., Au-Yeung, S.K., Lam, H., Kung, Y.Y., Zhang, X., van Vugt, J.M., Minekawa, R., Tang, M.H., Wang, J., Oudejans, C.B., Lau, T.K., Nicolaides, K.H., Lo, Y.M., 2011. Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study. BMJ 342, c7401.

Faas, B.H., Beuling, E.A., Christiaens, G.C., von dem Borne, A.E., van der Schoot, C.E., 1998. Detection of fetal RHD-specific sequences in maternal plasma. The Lancet 352, 1196.

Fan, H.C., Quake, S.R., 2007. Detection of aneuploidy with digital polymerase chain reaction. Analytical Chemistry 79, 7576–7579.

Fan, H.C., Blumenfeld, Y.J., Chitkara, U., Hudgins, L., Quake, S.R., 2008. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proceedings of the National Academy of Sciences of the United States of America 105, 16266–16271.

Hamada, H., Arinami, T., Kubo, T., Hamaguchi, H., Iwasaki, H., 1993. Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. Human Genetics 91, 427–432.

Hassold, T., Abruzzo, M., Adkins, K., Griffin, D., Merrill, M., Millie, E., Saker, D., Shen, J., Zaragoza, M., 1996. Human aneuploidy: incidence, origin, and etiology. Environmental and Molecular Mutagenesis 28, 167–175.

Lo, Y.M., Corbetta, N., Chamberlain, P.F., Rai, V., Sargent, I.L., Redman, C.W., Wainscoat, J.S., 1997. Presence of fetal DNA in maternal plasma and serum. The Lancet 350, 485–487.

Lo, Y.M., Tein, M.S., Lau, T.K., Haines, C.J., Leung, T.N., Poon, P.M., Wainscoat, J.S., Johnson, P.J., Chang, A.M., Hjelm, N.M., 1998. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. American Journal of Human Genetics 62, 768–775.

Lo, Y.M., Lun, F.M., Chan, K.C., Tsui, N.B., Chong, K.C., Lau, T.K., Leung, T.Y., Zee, B.C., Cantor, C.R., Chiu, R.W., 2007a. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. Proceedings of the National Academy of Sciences of the United States of America 104, 13116–13121.

Lo, Y.M., Tsui, N.B., Chiu, R.W., Lau, T.K., Leung, T.N., Heung, M.M., Gerovassili, A., Jin, Y., Nicolaides, K.H., Cantor, C.R., Ding, C., 2007b. Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. Nature Medicine 13, 218–223.

Mandel, P., Métals, P., 1948. Les acides nucléiques du sanguine chez l'homme. Comptes Rendus de l'Académie des Sciences de Paris 142, 241–243.

Old, R.W., Crea, F., Puszyk, W., Hulten, M.A., 2007. Candidate epigenetic biomarkers for non-invasive prenatal diagnosis of Down syndrome. Reproductive Biomedicine Online 15, 227–235.

Palomaki, G.E., Kloza, E.M., Lambert-Messerlian, G.M., Haddow, J.E., Neveux, L.M., Ehrich, M., van den Boom, D., Bombard, A.T., Deciu, C., Grody, W.W., Nelson, S.F., Canick, J.A., 2011. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. Genetics in Medicine 13, 913–920.

Papageorgiou, E.A., Fiegler, H., Rakyan, V., Beck, S., Hulten, M., Lamnissou, K., Carter, N.P., Patsalis, P.C., 2009. Sites of differential DNA methylation between placenta and peripheral blood: molecular markers for noninvasive prenatal diagnosis of aneuploidies. American Journal of Pathology 174, 1609–1618.

Papageorgiou, E.A., Karagrigoriou, A., Tsaliki, E., Velissariou, V., Carter, N.P., Patsalis, P.C., 2011. Fetal specific DNA methylation ratio permits non-invasive prenatal diagnosis of trisomy 21. Nature Medicine 17, 510–513.

Poon, L.L., Leung, T.N., Lau, T.K., Chow, K.C., Lo, Y.M., 2002. Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. Clinical Chemistry 48, 35–41.

Price, T.S., Regan, R., Mott, R., Hedman, A., Honey, B., Daniels, R.J., Smith, L., Greenfield, A., Tiganescu, A., Buckle, V., Ventress, N., Ayyub, H., Salhan, A., Pedraza-Diaz, S., Broxholme, J., Ragoussis, J., Higgs, D.R., Flint, J., Knight, S.J., 2005. SW-ARRAY: a dynamic programming solution for the identification of copy-number changes in

genomic DNA using array comparative genome hybridization data. Nucleic Acids Research 33, 3455–3464.

Rakyan, V.K., Down, T.A., Thorne, N.P., Flicek, P., Kulesha, E., Graf, S., Tomazou, E.M., Backdahl, L., Johnson, N., Herberth, M., Howe, K.L., Jackson, D.K., Miretti, M.M., Fiegler, H., Marioni, J.C., Birney, E., Hubbard, T.J., Carter, N.P., Tavare, S., Beck, S., 2008. An integrated resource for genome-wide identification and analysis of human tissue-specific differentially methylated regions (tDMRs). Genome Research 18, 1518–1529.

Tong, Y.K., Jin, S., Chiu, R.W., Ding, C., Chan, K.C., Leung, T.Y., Yu, L., Lau, T.K., Lo, Y.M., 2010a. Noninvasive prenatal detection of trisomy 21 by an epigenetic – genetic chromosome-dosage approach. Clinical Chemistry 56, 90–98.

Tong, Y.K., Chiu, R.W., Akolekar, R., Leung, T.Y., Lau, T.K., Nicolaides, K.H., Lo, Y.M., 2010b. Epigenetic – genetic chromosome dosage approach for fetal trisomy 21 detection using an autosomal genetic reference marker. PLoS One 5, e15244.

Tsaliki, E., Papageorgiou, E.A., Spyrou, C., Goumbaris, G., Kypri, E., Kyriakou, S., Sotiriou, C., Touvana, E., Keravnou, A., Karagrigoriou, A., Lamnissou, K., Velissariou, V., Patsalis, P.C., submitted for publication. MeDIP real time qPCR of maternal peripheral blood reliably identifies trisomy 21. Prenatal Diagnosis.

Tsui, N.B., Akolekar, R., Chiu, R.W., Chow, K.C., Leung, T.Y., Lau, T.K., Nicolaides, K.H., Lo, Y.M., 2010. Synergy of total PLAC4 RNA concentration and measurement of the RNA single-nucleotide polymorphism allelic ratio for the noninvasive prenatal detection of trisomy 21. Clinical Chemistry 56, 73–81.

Verweij, E.J., van den Oever, J.M., de Boer, M.A., Boon, E.M., Oepkes, D., 2012. Diagnostic accuracy of noninvasive detection of fetal trisomy 21 in maternal blood: a systematic review. Fetal Diagnosis and Therapy 31, 81–86.

Wright, C.F., Burton, H., 2009. The use of cell-free fetal nucleic acids in maternal blood for non-invasive prenatal diagnosis. Human Reproduction Update 15, 139–151.