Human Reproduction, Vol.36, No.11, pp. 2824–2839, 2021
Advance Access Publication on September 25, 2021 doi:10.1093/humrep/deab206

human reproduction

MINI REVIEW ***Developments in reproductive medicine and biology***

Liquid biopsy: state of reproductive medicine and beyond

Gaby Schobers [1,2,†], Rebekka Koeck [1,3,†], Dominique Pellaers[1,3], Servi J.C. Stevens [1], Merryn V.E. Macville[1], Aimée D.C. Paulussen[1,3], Edith Coonen[1,4], Arthur van den Wijngaard[1], Christine de Die-Smulders[1,3], Guido de Wert[5], Han G. Brunner[1,2,3], and Masoud Zamani Esteki [1,3,*]

[1]Department of Clinical Genetics, Maastricht University Medical Centre+, Maastricht, The Netherlands [2]Department of Human Genetics, Radboud University Medical Center, Nijmegen, The Netherlands [3]Department of Genetics and Cell Biology, GROW School for Oncology and Developmental Biology, Maastricht University, Maastricht, The Netherlands [4]Center for Reproductive Medicine, Maastricht University Medical Centre+, GROW School for Oncology and Developmental Biology, Maastricht University, Maastricht, The Netherlands [5]Faculty of Health, Medicine and Life Sciences, Department of Health, Ethics and Society, GROW School for Oncology and Developmental Biology, Maastricht University, Maastricht, The Netherlands

*Correspondence address. Department of Clinical Genetics, Laboratory of Cellular Genomic Medicine, Maastricht UMC+, P. Debyelaan 25, 6229 HX Maastricht, The Netherlands. Tel: +31-43-38-75306; E-mail: masoud.zamaniesteki@mumc.nl https://orcid.org/0000-0003-3909-0050

Submitted on October 2, 2020; resubmitted on August 6, 2021; editorial decision on August 13, 2021

ABSTRACT: Liquid biopsy is the process of sampling and analyzing body fluids, which enables non-invasive monitoring of complex biological systems *in vivo*. Liquid biopsy has myriad applications in health and disease as a wide variety of components, ranging from circulating cells to cell-free nucleic acid molecules, can be analyzed. Here, we review different components of liquid biopsy, survey state-of-the-art, non-invasive methods for detecting those components, demonstrate their clinical applications and discuss ethical considerations. Furthermore, we emphasize the importance of artificial intelligence in analyzing liquid biopsy data with the aim of developing ethically-responsible non-invasive technologies that can enhance individualized healthcare. While previous reviews have mainly focused on cancer, this review primarily highlights applications of liquid biopsy in reproductive medicine.

Key words: liquid biopsy / circulating cells / cell-free DNA / ART / IVF / spent embryo culture medium / non-invasive prenatal testing / preimplantation genetic testing / next generation sequencing / artificial intelligence

Introduction

Traditionally, studying pathogenesis commences with the sampling of a tissue or cytological specimen from the affected site of the human body. Such sampling usually requires invasive procedures, posing possible serious complications to the patient. Additionally, these procedures are inherently prone to incomplete representation of the affected tissue or cells (Gerlinger *et al.*, 2012) and require prior knowledge of the anatomical site of disease presentation. A promising alternative to invasive sampling are liquid biopsy techniques, which make use of circulating components in the body. Due to their non-invasive nature and representation of the tissue of origin, these emerging techniques offer a promising alternative to detect health- and disease-specific markers (Fig. 1).

Not all circulating components are equally abundant in body fluids, nor are they equivalent with respect to their size and biological properties. They range from rare, large circulating cells, e.g. circulating tumor cells (CTCs) and circulating trophoblastic cells, to more abundant, short cell-free nucleic acids (cfNAs), such as cell-free DNA (cfDNA), cell-free RNA (cfRNA) and circulating microRNA (miRNA). Furthermore, cfNAs do not only circulate in isolation, they can also be associated with protective protein complexes or encapsulated within extracellular vesicles (EVs). The accurate detection and characterization of low abundant circulating components in liquid biopsy still poses a challenge, especially as they are often dispersed among material originating from multiple tissues (Sun *et al.*, 2015). Therefore, sufficient sampling and sophisticated computational approaches are required to generate reliable results for clinical reports.

While previous reviews about liquid biopsy have primarily focused on cancer, here we highlight its importance and potential in reproductive medicine by: describing past liquid biopsy component discoveries; summarizing technological advances in the field; showcasing potential

[†]These authors are joint first authors.

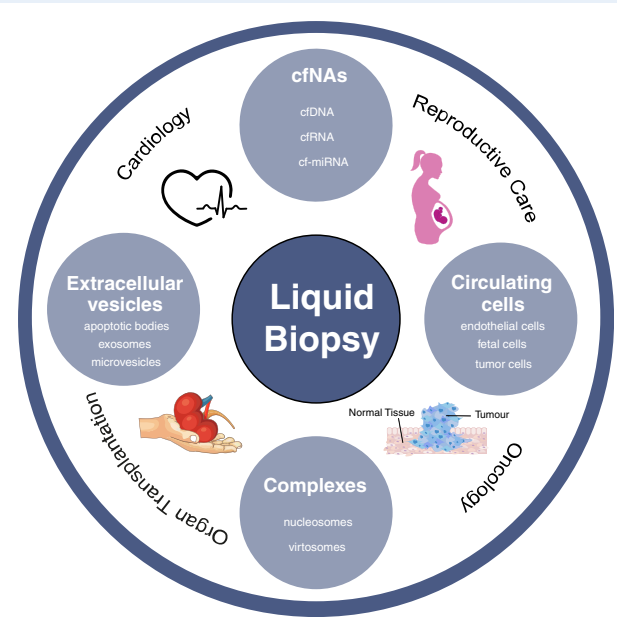

Figure 1. Dissecting the liquid biopsy. Analysis of circulating components within body fluids can be used for non-invasive disease detection and monitoring in several medical disciplines including cardiology, oncology, transplant and reproductive medicine. Different circulating components, such as cells, cell-free nucleic acids (cfNAs) and extracellular vesicles (EVs), are utilized for different tests. cfDNA, cell-free DNA; cfRNA, cell-free RNA; cf-miRNA, cell-free micro RNA.

applications of those technologies in reproductive medicine; highlighting the importance of artificial intelligence (AI); and discussing the ethical principles that these novel possibilities may engender.

Detection of different liquid biopsy components

Circulating cells

CTCs, which were first described 151 years ago (Ashworth, 1869) (Fig. 2), can be characterized based on their size and cell-surface marker expression using size-based membrane filters and cell-sorting techniques, such as CellSearch (Adams *et al.*, 2015), the size of epithelial tumor cells (ISET) method (Mazzini *et al.*, 2014), CellSieve (Adams *et al.*, 2015), ScreenCell (Freidin *et al.*, 2014) and other microfluidic systems (Fig. 3, Table I). In principle, microfluidic systems perform electric charge-, density-, or size-based separation (Warkiani *et al.*, 2016). For instance, size-sorting microfluidic chips are designed to capture CTCs, which are larger (~17–52 µm) than leukocytes (~7–15 µm) and erythrocytes (~6–8 µm) (Neoh *et al.*, 2018). Similarly, circulating fetal trophoblastic cells, which were first discovered in the maternal circulation in 1893 (Schmorl, 1893), can be isolated by the ISET method, differentiating cytotrophoblast-like cells (~14.3–30 µm) and syncytiotrophoblast-like cells (~44–60 µm) (Vona *et al.*, 2002). In addition, other circulating fetal cells (CFCs) such as fetal erythroblasts, lymphocytes and granulocytes have been found in maternal blood (Zipursky *et al.*, 1959; Krabchi *et al.*, 2001). Efficient isolation of CFCs from maternal blood can be achieved by their enrichment using a panel of selective cell expression markers (Fig. 3Fi), such as CD105 and CD141 (Hatt *et al.*, 2014) or GB17, GB21 and GB25 (Bruch *et al.*, 1991), or by depleting their trophoblast-marker negative maternal counterparts (Fig. 3Fii). CFCs can be used for cell-based non-invasive prenatal testing (NIPT) (Vossaert *et al.*, 2019). Upon isolation, these cells provide a pure source of fetal genomic DNA. However, the main challenge is that CFCs are exceedingly rare, approximately 1–2 cell(s) per ml of maternal blood (Krabchi *et al.*, 2001), requiring a large volume of the maternal blood to perform this test. Even though cell-based NIPT enables enrichment of fetal cells and pure fetal copy number variation (CNV) detection (Breman *et al.*, 2016; Kølvraa *et al.*, 2016; Vossaert *et al.*, 2019), the extracted DNA from those fetal cells should be whole-genome amplified before genome sequencing; a process that introduces many artifacts, including allelic drop out and preferential amplification (see Sequencing section). Nevertheless, single-cell sequencing methods could alleviate this problem and are now validated for clinical use (Vossaert *et al.*, 2019).

Circulating cell-free nucleic acids

cfNAs, including cfDNA and RNA, were first described in 1948 (Mandel and Metais, 1948). They originate from cultivated cells, non-malignant somatic tissues, tumors and embryos or fetuses and are released when cells undergo necrosis or apoptosis. cfNAs can be characterized based on their length, physical size, surface molecules, electric charge and density (Fig. 3).

Cell-free DNA

In 1997, the presence of cell-free fetal DNA (cffDNA), originating from the placental trophoblast (Alberry *et al.*, 2007), was reported in the maternal circulation (Lo *et al.*, 1997). cffDNA that are released by apoptotic trophoblast cells within the fetal compartment of the placenta can be used for nucleic acid-based NIPT (Tjoa *et al.*, 2006), representing the fetus but not completely. This is due to confined placenta mosaicism (CPM), i.e. the presence of chromosomally abnormal cells in the placenta but not in the fetus. CPM can lead to false-positive cfDNA-based NIPT, which is now widely used. In contrast to pure fetal DNA from CFCs, cffDNA is fragmented and mixed with maternal DNA, which makes it even more challenging to identify true submicroscopic CNVs. A recent study showed that >70% of large CNVs (>10 kb) are confined to the placenta (Zamani Esteki *et al.*, 2019), this is well below the detection limit of cfDNA-based approaches and may be misinterpreted with maternal CNVs (Kotsopoulou *et al.*, 2015), including malignancies (see Application section). The size distribution of maternal serum cfDNA can be used for size- and origin-based diagnostic approaches (Fig. 3G), as maternal cfDNA fragments are, on average, longer (166 bp) than cffDNA fragments (143 bp) (Lo *et al.*, 2010). cfDNA is also detectable in blastocoel fluid (BF) of human embryos and in spent IVF culture medium, enabling minimally- and non-invasive genetic testing, respectively (Assou *et al.*, 2014). Furthermore, the presence of mitochondrial DNA (mtDNA) in the embryo's culture medium has been associated with fragmentation of the embryo caused by apoptosis or necrosis (Stigliani *et al.*, 2013) (see Application section).

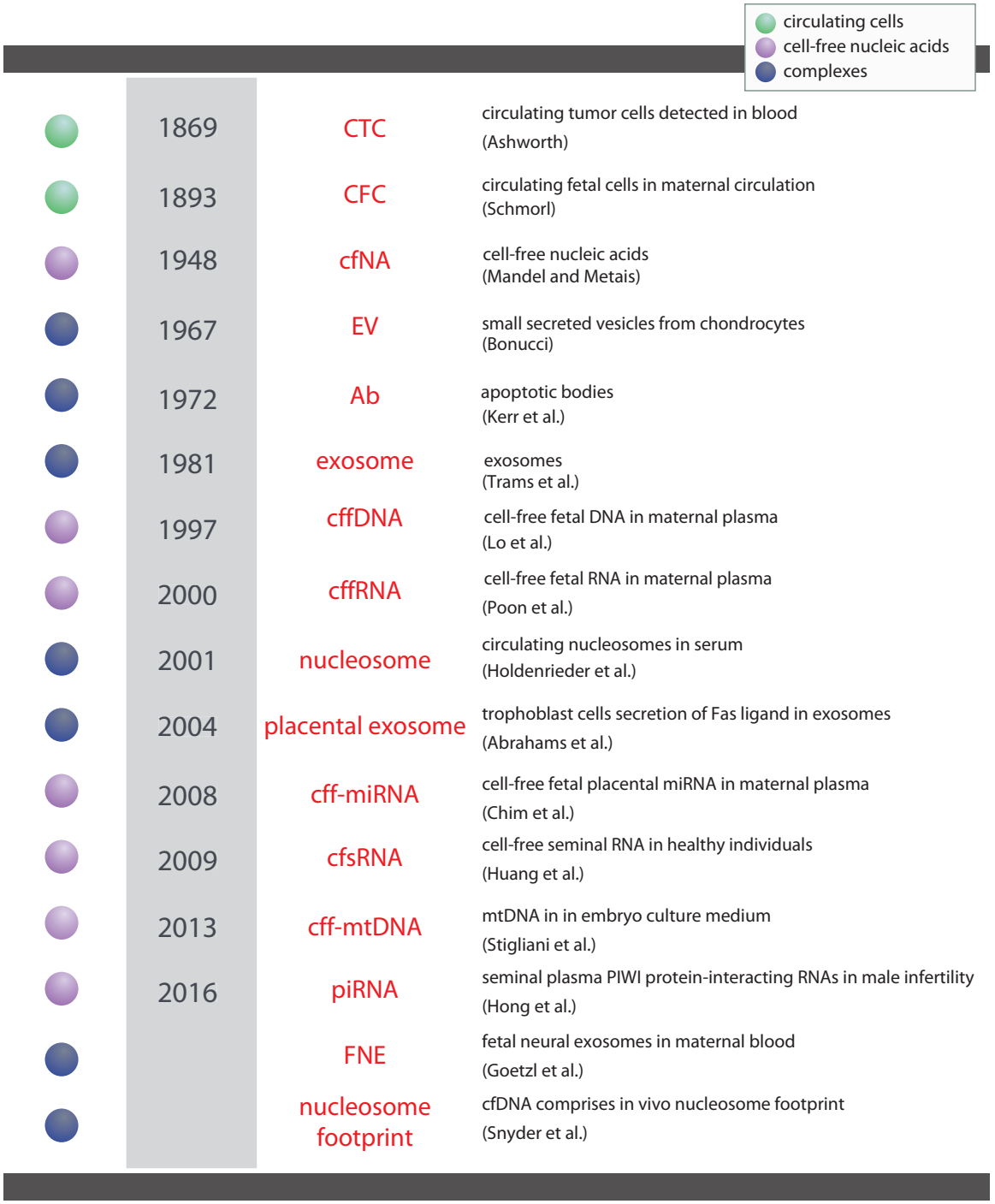

Figure 2. A timeline capturing the discovery of liquid biopsy components. About a century after the discovery of circulating cells (green), cell-free nucleic acids (cfNAs) (purple) were observed. Their clinical application was not established until decades later but sparked many further discoveries relating to cfNAs, as well as extracellular vesicles (EVs) and other complexes (blue) that can carry them, in rapid succession. CTC, circulating tumor cells; CFC, circulating fetal cells; Ab, apoptotic bodies; cffDNA, cell-free fetal DNA; cffRNA, cell-free fetal RNA; cff-miRNA, cell-free fetal micro RNA; cfsRNA, cell-free seminal RNA; cff-mtDNA, cell-free fetal mitochondrial DNA; piRNA, PIWI-interacting RNA; FNE, fetal neural exosomes.

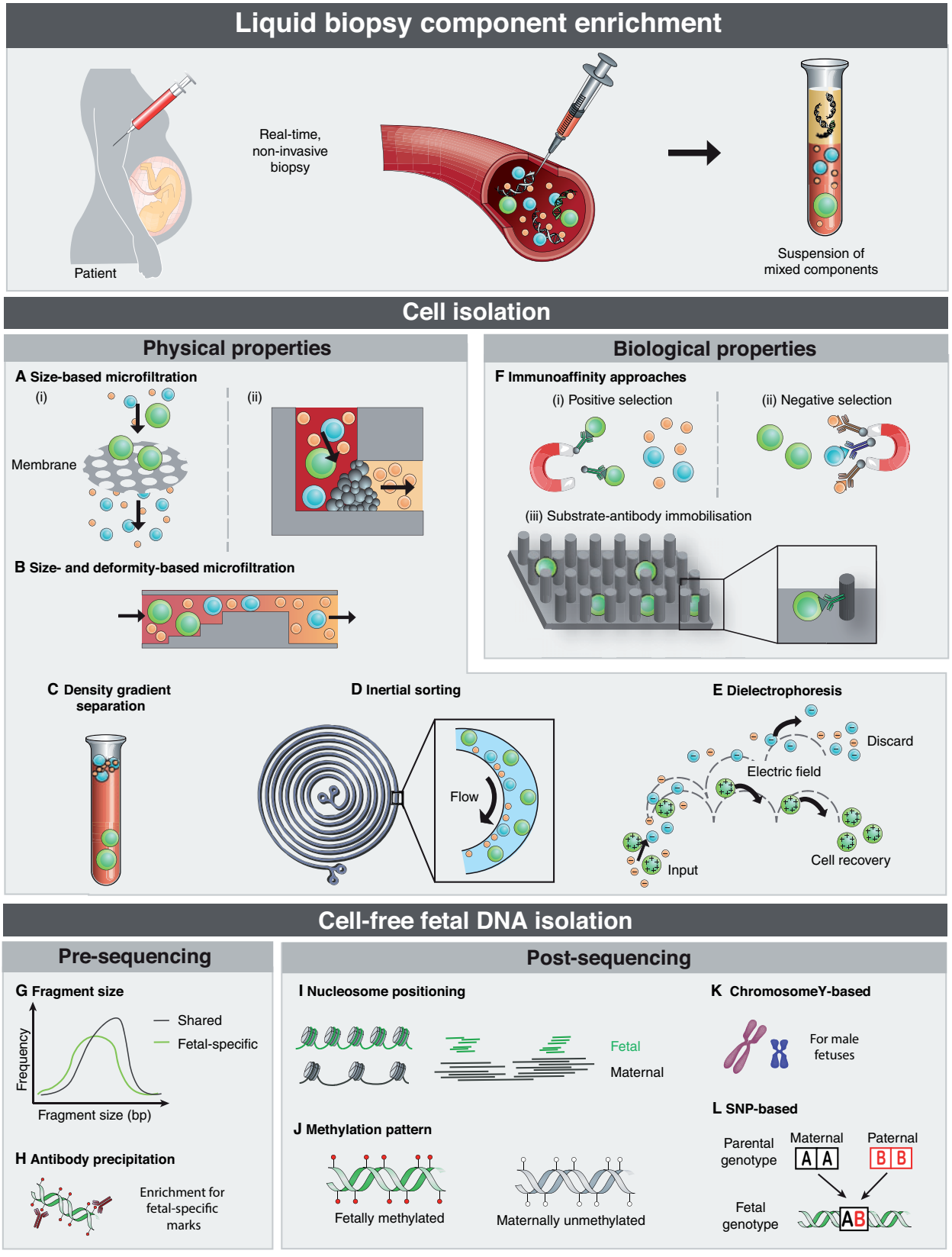

Figure 3. Enrichment of liquid biopsy components. To isolate a component of interest from the mixed liquid biopsy suspension, a variety of techniques are employed. Methods used to enrich for specific cell populations, such as epithelial, tumor, or fetal cells, are based on either, or both, physical or biological characteristics of the cells of interest. Enrichment of cells of interest or depletion of unwanted cells using physical properties is

Cell-free RNA

Since the detection of cell-free fetal RNAs in the blood stream of pregnant women in 2000 (Poon *et al.*, 2000), most studies have focused on placental small non-coding RNAs, such as miRNAs (Chim *et al.*, 2008) and especially those contained within protective exosomes (Huang *et al.*, 2013). This is because of their relatively greater stability and abundance as compared with mRNA. Placental miRNAs can serve as potential biomarkers for pregnancy complications such as preeclampsia and intra-uterine growth retardation, by influencing gene expression levels related to placental development

Table I Methods used in liquid biopsy processing.

Component		Enumeration	Genomics	Transcriptomics	Epigenomics	Single cell
Cells	CTC	+	+	+	+	+
	CFC	+	+	+	+	+
cfNAs	cfDNA	−	+	−	+	−
	cffDNA	−	+	−	+	−
	cfRNA	−	+	+	−	−
	cffRNA	−	+	+	−	−
	miRNA	−	+	+	+	−
Extracellular vesicles and other complexes	Exosome	−	+	+	−	−
	Small vesicles	−	+	−	−	−
	ABs	−	−	−	−	−
	Nucleosomes	−	−	−	+	−
Methods		CellSearch (Adams *et al.*, 2015) ISET (Mazzini *et al.*, 2014) CellSieve (Adams *et al.*, 2015) ScreenCell (Freidin *et al.*, 2014)	ddPCR (Chen *et al.*, 2013) BEAMing (Chen *et al.*, 2013) Tam-Seq (Forshew *et al.*, 2012) WGS (Leary *et al.*, 2012)	qRT-PCR (Byron *et al.*, 2016) Microarray (Byron *et al.*, 2016) RNA-seq (Max *et al.*, 2018)	RRBS (Laird, 2010) Targeted BS (Laird, 2010) WGBS (Laird, 2010)	MALBAC (Hou *et al.*, 2013; Huang *et al.*, 2014) MDA (Huang *et al.*, 2015) DOP-PCR (Chappell *et al.*, 2018) DR-seq (Dey *et al.*, 2015) G&T-seq (Macaulay *et al.*, 2015) scNMT-seq (Clark *et al.*, 2018) scCOOL-seq (Li *et al.*, 2018)

AB, apoptotic bodies; BEAMing, bead, emulsion, amplification and magnetics; CFC, circulating fetal cell; cfDNA, cell-free DNA; cffDNA, cell-free fetal DNA; cffRNA, cell-free fetal RNA; cfRNA, cell-free RNA; CTC, circulating tumor cell; ddPCR, droplet digital PCR; DOP-PCR, degenerate oligonucleotide-primed PCR; DR-seq, gDNA and mRNA sequencing; G&T-seq, genome and transcriptome sequencing; MALBAC, multiple annealing and looping-based amplification cycles; MDA, multiple displacement amplification; miRNA, microRNA; qRT-PCR, quantitative reverse transcription PCR; RRBS, reduced representation bisulfite sequencing; scCOOL, single cell chromatin overall omic-scale landscape sequencing; scNMT, single cell nucleosome, methylation and transcription sequencing; Tam-Seq, tagged-amplicon deep sequencing; WGBS, whole-genome bisulfite sequencing; WGS, whole genome sequencing.

Figure 3. Continued

achieved by size-based microfiltration, using membranes (**Ai**) or packed beads (**Aii**), size- and deformity-based microfiltration (**B**), density gradient centrifugation (**C**), inertial sorting, based on cell size and hydrodynamics (**D**), or dielectrophoresis (**E**). Other common enrichment methods use cell-specific markers to separate cells based on their biological properties (**Fi**). By negative selection, a sample can be depleted of unwanted cells using the same approach (**Fii**). These capture agents can, for instance, be bound by magnetic particles or located on microchips (**Fiii**). The characteristics of cffDNA are used to enable enrichment for cffDNA before sequencing or determination of the fetal fraction after the maternal blood sample has been sequenced. Pre-sequencing (NGS library preparation), cfDNA fragment sizes can be used for size selection to discriminate cffDNA from the maternal cfDNA (**G**). Fetal-specific marks can also be used for affinity-based enrichment (**H**). Post-sequencing, the proportion of cffDNA sized fragments, nucleosome positioning (**I**) and methylation patterns (**J**) are correlated with the fetal fraction. Additionally, using parental genotype information, fetal specific alleles can be detected and used for the estimation of the fetal fraction (**K** and **L**). NGS; next generation sequencing, SNP; single nucleotide polymorphism.

(Awamleh *et al.*, 2019). However, characterizing miRNAs is challenging due to their short length (~22 nucleotides) and high level of homology, which complicates the use of primers. This obstacle has been overcome by the detection of several recognition elements that can be employed in electrochemical- or optical-based miRNA detection (Tian *et al.*, 2015). To characterize male-factor infertility, cfRNA (Huang *et al.*, 2009) and piwi-interacting RNA (Hong *et al.*, 2016) can be isolated from semen.

Extracellular vesicles and other complexes

In 1967, small secreted vesicles were first reported (Bonucci, 1967). EVs, such as apoptotic bodies (ABs) and exosomes, can be distinguished by their distinct characteristics. For example, exosomes (Trams *et al.*, 1981), which arise upon fusion of internal multivesicular endosomes with the plasma membrane, are the smallest EVs (~30–200 nm diameter) (Pegtel and Gould, 2019). Placental-derived exosomes (Abrahams *et al.*, 2004) identified in maternal plasma could serve as biomarkers for the diagnosis and prognosis of preeclampsia as they are elevated in pre-symptomatic pregnancies, which subsequently may develop preeclampsia. Recently, specific fetal neural exosomes (FNEs) have been isolated from maternal plasma during pregnancy (Goetzl *et al.*, 2016). FNEs can potentially be used as a diagnostic tool to detect early signs of fetal neurological disease and are correlated with fetal exposure to alcohol (Goetzl *et al.*, 2019).

ABs (Kerr *et al.*, 1972), containing degraded DNA, generated during apoptosis, are the largest EVs (4000–5000 nm diameter) (Tixeira *et al.*, 2017). Alternatively, cfNAs can circulate in association with protective protein complexes, such as nucleosomes (Holdenrieder *et al.*, 2001). Importantly, from the exact spacing of nucleosomes, the tissue of origin of a cfNA fragment can be determined (Snyder *et al.*, 2016). For instance, maternal cfDNA is predominantly cleaved with the linker region intact while cffDNA is cleaved at the border with or within the nucleosome (Shi *et al.*, 2020) (Fig. 3I). Different EV types can be detected by centrifugation, (agglutination-)precipitation, or ultracentrifugation after size-exclusion. Exosomes can also be captured by immunoaffinity-beads and microfluidic chip methods and can be enriched using antibody-based label or label-free exosome arrays (Ko *et al.*, 2016).

Sequencing of liquid biopsy components

Sequencing cell-free nucleic acids

The introduction of next-generation sequencing (NGS) has enabled the detection of genomic variants, such as point mutations, CNVs and structural aberrations (Goodwin *et al.*, 2016) across the fetal genome. Furthermore, NGS offers a reliable platform for multi-faceted analysis of cfNAs, including exome (Butler *et al.*, 2015), (epi)genome (Laird, 2010; Leary *et al.*, 2012) and transcriptome (Max *et al.*, 2018) analyses (Table I).

One NGS approach, to detect mutant alleles that are present in at least 2% of circulating tumor DNA fragments, is tagged-amplicon deep sequencing (Forshew *et al.*, 2012), which is based on targeted resequencing of a panel of specific low-frequency mutations. Rarer mutant alleles can be detected with other targeted approaches, such as droplet digital PCR or parallel beads, emulsion, amplification and magnetics PCR (Chen *et al.*, 2013).

A challenge in NGS data analysis is tackling background error noise, which can occur when 'jackpot' mutations arise after errors in the first PCR cycle and by preferential amplification. This can be solved by computational approaches that correct for GC content or by assuming a diploid genome as a baseline, e.g. GC and median corrections, respectively (Bayindir *et al.*, 2015). Molecular barcoding, unique molecular identifiers (UMIs) and circle sequencing can also reduce these errors. For instance, UMIs facilitate the grouping of sequence reads according to DNA molecule, thereby distinguishing between true variants and artifacts. Similarly, circle sequencing reduces sequencing error rates by circularization of single-stranded DNA fragments, followed by inverse PCR or rolling circle amplification (Lou *et al.*, 2013). Thus, both robust computational pipelines and wet-lab protocols are key to produce accurate results.

NGS-based methods can also be applied to study the epigenome of cfNAs and to characterize cfRNAs. The epigenome, and specifically DNA methylation, can be used to ascertain the tissue-of-origin of cfDNA (Chim *et al.*, 2005). DNA methylation is most commonly examined using bisulfite treatment of DNA, which converts unmethylated cytosine residues to uracil. Subsequent methylome profiling can be performed in three forms (Laird, 2010): (i) reduced representation bisulfite sequencing, which is untargeted but enriches for CpG-rich fragments; (ii) targeted capture of bisulfite-converted DNA, either by array or padlock capture; and (iii) whole-genome bisulphite sequencing (WGBS). WGBS, for instance, has been used on cfDNA to uncover placenta hypomethylation, which might aid in cffDNA enrichment (Jensen *et al.*, 2015). The transcriptome can be profiled by technologies such as quantitative reverse transcription-PCR, microarray and RNA sequencing (Byron *et al.*, 2016). Improved NGS techniques have facilitated the isolation of nanogram quantities of cfRNA from serum and plasma. Plasma- and serum-specific profiles can then be discerned by probing different RNA forms, such as miRNAs, mRNA and tRNAs (Max *et al.*, 2018).

Sequencing circulating (single) cells

Advances in cell isolation, whole-genome amplification (WGA) and NGS have contributed to the emergence of single-cell genomics. DNA sequencing of scarce circulating cells requires WGA as they only possess a small amount of DNA (~7 picogram). Multiple annealing and looping-based amplification cycles, multiple displacement amplification and degenerate oligonucleotide-primed PCR are the most commonly used WGA methods (Huang *et al.*, 2015). Recently, techniques for simultaneous analysis of multiple omic layers in single cells, e.g. genomic DNA and mRNA in DR-seq (Dey *et al.*, 2015), the genome and transcriptome in G&T-seq (Macaulay *et al.*, 2015), single-cell Nucleosome, Methylation and Transcription in scNMT-seq (Clark *et al.*, 2018) and (improved) single-cell Chromatin Overall Omic-scale Landscape Sequencing in (i)scCOOL-seq (Gu *et al.*, 2019) that can detect chromatin state, nucleosome positioning, methylation, CNV and ploidy, have emerged. Such bi- or multi-layer omic assays are of paramount importance to establish the interplay and connectivity (a.k.a. molecular circuitry) between different molecular layers by facilitating their direct side-by-side comparison.

Robust analysis of any single-cell omic-layer requires highly efficient capture and enrichment techniques to reduce technical artifacts. Specifically, WGA is prone to technical errors, such as non-linear amplification and insufficient coverage (Navin, 2015). Analysis of the single-cell transcriptome further introduces the challenge of recovering the full-length cDNA, quantification of translational activity and sense and antisense transcript discrimination (Tang et al., 2011).

Applications of liquid biopsy in reproductive medicine

Liquid biopsy has potential and already realized applications in different fields. It is now evident that cfDNA is highly representative of genomic DNA as it shares many of its features (Beck et al., 2009; van der Vaart et al., 2009). Interestingly, cfDNA has a short half-life (4 min to 2.5 h), as it is rapidly filtered out of the circulation by the liver, spleen and kidney (Khier and Lohan, 2018). Caution is warranted using quantitative analysis methods as suboptimal extraction processes can affect the quantity of isolated cfDNA. Serum contains a relatively high concentration of cfDNA, but is less informative, as a greater proportion of serum cfDNA originates from leucocyte lysis. For this reason, DNA is preferentially extracted from plasma (Lee et al., 2001). Importantly, cfNAs can be traced to their tissue of origin, which makes them informative biomarkers for assisted reproductive technology (ART) (Wu et al., 2015; Snyder et al., 2016; Liu et al., 2017).

Assisted reproductive technology

In-vitro fertilization

Embryo selection procedures in IVF aim to identify good quality embryos with the highest implantation potential. Follicular fluid (FF), which influences maturation of follicles and oocyte growth *in vivo*, contains cfDNA of apoptotic granulosa cells and is sampled as part of the IVF oocyte retrieval process. Low levels of cfDNA in FF samples are significantly correlated with low embryo fragmentation rate and are indicative of high-quality embryos (Scalici et al., 2014). FF also contains cell-free mitochondrial DNA (cf-mtDNA), which can be quantified to predict embryo viability (Stigliani et al., 2014). Cumulus cells (CCs), surrounding the oocyte during its development, have been shown to increase the quantities of cf-mtDNA in the IVF culture medium if mitochondrial dysfunction is present (Kansaku et al., 2018). The effect of mitochondrial dysfunction is being studied with the hope of gaining further insights into embryo quality and being able to predict the developmental competence and implantation potential of the embryo. CC gene expression is also a valid biomarker of oocyte quality, as the expression of specific genes in CCs correlates with embryo potential and pregnancy outcome (Assou et al., 2008). In addition, a novel non-invasive metabolomics approach for embryo selection has been established, which identified 60 metabolomic biomarkers of euploidy and aneuploidy in spent IVF culture medium (SCM) (Cabello-Pinedo et al., 2020), demonstrating the power of metabolomics in IVF as a non-invasive selection approach.

Male infertility

Presence of higher levels of cell-free seminal DNA (cfs-DNA) is associated with azoospermia (Li et al., 2009), i.e. absence of sperm in the semen. In these cases, testicular sperm can be utilized for ICSI during IVF. cfs-DNA is detectable in both normal and azoospermic semen samples. Epigenetic analysis of the same cfs-DNA also identifies differences, such as hypermethylation, that are associated with hypospermatogenesis (Wu et al., 2013). Additionally, cf-RNA can be isolated from semen (Huang et al., 2009), such that analysis of cfs-mRNA accurately distinguishes between non-obstructive and obstructive azoospermia (Li et al., 2012).

Preimplantation genetic testing

Preimplantation genetic testing (PGT) is an ART that prevents transmission of genetic disorders to the offspring (Handyside et al., 1990). Currently, testing is primarily either conducted on a single blastomere taken from a cleavage-stage embryo, or on a few trophectoderm (TE) cells taken from a blastocyst, both of which are obtained through invasive biopsy methods that may be harmful to the embryo (Kuliev and Rechitsky, 2017). PGT is offered for monogenic disorders (Wu et al., 2015; Liu et al., 2017), structural rearrangements and aneuploidies (PGT-A) (Kuliev and Rechitsky, 2017; Liu et al., 2017). However, the clinical utility of PGT-A in its current form, i.e. only determining the number of chromosomes, is still under debate (Mastenbroek and Repping, 2014; Vermeesch et al., 2016).

Minimally and non-invasive preimplantation genetic testing

Following *in vitro* culture, the transfer of embryos can be postponed by cryopreservation using vitrification (Zhu et al., 2011), a process that requires collapse of the expanded blastocyst. If not occurring spontaneously, artificial shrinkage can be induced by BF microsuction (Chen et al., 2005). A BF biopsy is a minimally invasive procedure as it does not involve removal of cells from the embryo and thus should be less harmful to the embryo as compared with standard cell-biopsy-based PGT methods. The non-invasive alternative would be embryonic-derived cfDNA in SCM (Galluzzi et al., 2015; Shamonki et al., 2016; Xu et al., 2016; Feichtinger et al., 2017). cfDNA shed into the blastocyst cavity and SCM has been proposed to be derived from apoptotic cells (Palini et al., 2013; Gianaroli et al., 2014). However, current studies suggest that other mechanisms, besides apoptosis and necrosis, may be involved in DNA release from the inner cell mass and TE in BF and SCM. Recently, it was demonstrated that the amount of cffDNA in BF and SCM or the concordance rates of NGS results were similar for both moderate/low and good quality blastocysts (Kuznyetsov et al., 2020). Nevertheless, current media-based non-invasive PGT methods remain inferior to standard cell-biopsy-based invasive PGT methods, reaching a maximum of 80–90% concordances between non-invasive PGT and standard PGT (Lane et al., 2017; Ho et al., 2018), thus leaving ~10% of samples of which no informative results could be given. Recently, the combination of cffDNA from BF and SCM was shown to reach an overall concordance rate of 88/90 (97.8%) for euploidy/aneuploidy status between minimally invasive PGT-A and TE biopsy samples (Kuznyetsov et al., 2020). This implies that the combination of BF and SCM shows promise for the clinical application of minimally invasive PGT. One of the remaining challenges is maternal cfDNA contamination in the culture medium that arises from the presence of, for instance, maternal CCs (Hammond et al., 2017; Vera-Rodriguez et al., 2018). It has been suggested that maternal contamination can be minimized by omitting the cell lysis step recommended in the procedure of WGA (Kuznyetsov et al., 2020).

Next-generation preimplantation genetic testing

Traditional PGT methods are based on targeted multiplex PCR or fluorescent *in situ* hybridization (Sermon *et al.*, 2004). However, high throughput genomic technologies, such as single-nucleotide polymorphism (SNP)-array and NGS-based haplotyping, are revolutionizing PGT (Handyside *et al.*, 2010; Natesan *et al.*, 2014; Zamani Esteki *et al.*, 2015; Backenroth *et al.*, 2019; Masset *et al.*, 2019). The addition of parental haplotyping can offer further valuable insights for PGT-A (Zamani Esteki *et al.*, 2015). A haplotype represents consecutive alleles that are inherited together on a stretch of DNA, indicating which parts of homologous parental chromosomes are transmitted to the embryo. As such, the mechanistic origin of aneuploidies, i.e. meiotic or mitotic, can be determined. This is vital information, because aneuploidies originating during meiosis are likely to affect all the cells of an embryo, and hence the fetus, leading to the aneuploidy-associated phenotype or miscarriage. On the other hand, aneuploidies with a mitotic origin are only present in a fraction of embryonic cells and can therefore be outgrown by euploid cells, leading to successful pregnancies with genetically normal infants (Zamani Esteki *et al.*, 2019). Such mitotic aneuploidies may arise because of chromosome instability, which is commonly seen in preimplantation human embryos (Vanneste *et al.*, 2009; Zamani Esteki *et al.*, 2015).

Prenatal genetic testing

Traditionally, prenatal genetic testing involves invasive chorionic villus or amniotic fluid sampling. However, the non-negligible risk of pregnancy loss associated with these techniques urged the need for non-invasive alternatives. Although plasma levels of cffDNA increase during pregnancy, its isolation remains challenging due to its relative low abundance (Lo *et al.*, 1998). The presence of fetal DNA can be confirmed by Y-chromosome markers (Lo *et al.*, 1999) (Fig. 3K). However, this is only the case in pregnancies with male fetuses and is unreliable due to false negatives caused by insufficient sampling. Alternatively, cffDNA can be recognized by the presence of paternally inherited short tandem repeats (Pertl *et al.*, 2000) (Fig. 3L). Furthermore, distinct DNA methylation patterns of placental and maternal genes can be used to establish the cellular origin of cfDNA fragments (Chim *et al.*, 2005) (Fig. 3J). Stable mRNA transcripts from placenta-expressed genes have also been used as markers to detect pregnancy pathologies and for non-invasive fetal sex determination (Mersy *et al.*, 2015).

Despite challenges relating to isolation and characterization of cffDNA from the maternal circulation, NIPT has been introduced into clinical practice (Bianchi *et al.*, 2014; Vermeesch *et al.*, 2016), not just for high-risk pregnancies but also as part of a screening program for all pregnancies (van der Meij *et al.*, 2019). Currently, three forms of NIPT can be carried out, including NIPT for aneuploidy, structural rearrangements and monogenic disorders (NIPT-M) (Lam *et al.*, 2012; Lv *et al.*, 2015; Yin *et al.*, 2018; Zhang *et al.*, 2019). Initially, the median increase of fetal DNA concentration was used as a marker of trisomy 21 (Lo *et al.*, 1999). Subsequently, chromosome-specific markers have been introduced, allowing the detection of chromosomal anomalies using either the allelic ratios from SNP genotyping (Lo *et al.*, 2007a), e.g. CNV detection in alpha-thalassemia (Ge *et al.*, 2013), or the transcriptome unique to the fetus (Lo *et al.*, 2007b). Advances in the technology even permit the detection of fetal single-gene defects from maternal plasma. In most instances, NIPT-M involves targeted sequencing of genes of interest (Zhang *et al.*, 2019). For example, targeted enrichment by solution-based hybridization followed by sequencing and haplotyping of the β-globin gene region can detect mutations and diagnose β-thalassemia (Lam *et al.*, 2012), and targeted massively parallel sequencing provides early prenatal diagnosis of fetuses at risk for congenital adrenal hyperplasia (New *et al.*, 2014). Further improvements have come from the introduction of single-molecule amplification and resequencing technology (Lv *et al.*, 2015) and new algorithmic methods (Yin *et al.*, 2018; Rabinowitz *et al.*, 2019) in combination with isolating circulating cells.

NIPT samples are typically processed using shallow sequencing protocols, such as WISECONDOR (Straver *et al.*, 2014; Bayindir *et al.*, 2015; Raman *et al.*, 2019), which can detect aneuploidies and large CNVs using inter-chromosomal read count comparison. While these methods have high analytical accuracy, detected chromosomal abnormalities still need to be confirmed by invasive testing (Bianchi *et al.*, 2014; Bayindir *et al.*, 2015; van der Meij *et al.*, 2019). This is a biological rather than a technical problem, as CPM is observed in 1–2% of pregnancies (Kalousek and Vekemans, 1996). Besides CPM-related false positive trisomies of autosomes, NIPT has limited utilities for sex chromosome aneuploidies, for instance low-level mosaicism of chromosome X that is due to age-related loss of chromosome X in women (Russell *et al.*, 2007) can lead to false positive Turner syndrome (45, X) diagnoses (Wang *et al.*, 2020). Thus, the use of CFCs can solve misdiagnoses that are caused by fetal (e.g. CPM) or maternal (e.g. loss of chromosome X) mosaicisms. For NIPT-M, haplotyping-based analysis methods, e.g. relative haplotype dosage analysis (Lo *et al.*, 2010), have been implemented to trace allelic inheritance. However, current haplotyping methods require high-coverage NGS, and necessitate parental samples to define heterozygous parental SNPs whose relative representation can be sought in the fetal genome (Fan *et al.*, 2012). Haplarithmisis is a more sophisticated genome-wide haplotyping method that makes use of continuous B-allele fractions, instead of error-prone discrete SNP genotypes, to determine copy number states (Zamani Esteki *et al.*, 2015) alongside the parental and segregation origin of genomic alterations in cffDNA (Che *et al.*, 2020).

It is now evident that NIPT has the potential to detect maternal malignancies. cfDNA from malignant cells can be shed into the blood stream (Bianchi, 2018). As such, it provided proof-of-principle of liquid biopsy for cancer screening in large populations (Lenaerts *et al.*, 2021). Given the fact that the population screened in NIPT is relatively young, the incidence of maternal malignancies is low. Large cohort studies estimate the frequency with which malignancies can be detected with the current NIPT protocol is in the range of about 1:10 000 (Bianchi *et al.*, 2015; Dharajiya *et al.*, 2018; van der Meij *et al.*, 2019). The malignancies in NIPT as reported in literature include (amongst others) leukemia or lymphoma, breast and cervical carcinoma (Ji *et al.*, 2018). However, it may be difficult to pinpoint where a putative tumor may be located based solely on genomic aberrations. Furthermore, a substantial number of suspicious NIPT findings are not confirmed by subsequent diagnostic tests or physical examinations. This may be due to occult malignancies or benign proliferations that are below the level of detection of current diagnostic techniques or due to placenta abnormalities. In addition, the aberrant NIPT signals may be derived from benign clonal proliferations, e.g. leiomyoma (Dharajiya *et al.*, 2018) for

which it is questionable whether detection during pregnancy is beneficial (Bianchi, 2018).

As data are scarce, there are currently no evidence-based guidelines for counseling and clinical follow-up after a NIPT result putatively indicating a malignancy. A multidisciplinary collaboration that includes genetic counselors, oncologists, clinical laboratory geneticists and gynecologists is deemed crucial for accurate patient management (Giles et al., 2017; Smith et al., 2017; Bianchi, 2018). Although studies thus far look promising, they did not investigate the assumed clinical benefits of earlier detection versus the burden that a NIPT finding may impose on the woman, and there has been no follow-up of women screened negative (Bianchi, 2018). As with all screening programs, the question that remains is whether earlier detection of malignancy in a pregnant population ultimately leads to better clinical outcomes for both mother and child.

Future directions

Diagnostic, prognostic and therapeutic values

Liquid biopsy is an emerging field with numerous important applications (Table II). In particular, these techniques are invaluable for screening practices. For example, NIPT can be used in place of conventional invasive testing procedures that are associated with a risk of miscarriage (van Schendel et al., 2017). Additionally, liquid biopsies can be used for risk-free screening of asymptomatic individuals, promising reduced morbidity and mortality associated with conditions where treatment success diminishes with disease progression. For instance, earlier diagnosis and more accurate monitoring of preeclampsia (Wahid et al., 2018) and other pregnancy-related pathologies (Pernemalm et al., 2019) may be possible using liquid biopsies, thereby facilitating the timely initiation of appropriate treatment and a reduction of complications. Liquid biopsy may also be implemented to predict progression in other diseases, as is the case in heart failure where the circulating long non-coding RNA LIPCAR can be used to predict survival (Kumarswamy et al., 2014). Similarly, treatment response could be assessed using cfDNA monitoring as already illustrated in transplantation medicine (Burnham et al., 2017). Circulating components have even shown promise for therapeutic applications. For instance, EVs have been developed as highly biocompatible, stable, tissue-targeted drug delivery systems (Meng et al., 2020).

Artificial intelligence

Emerging (ultra-)sensitive technologies and their introduction in healthcare systems generate extensive datasets, necessitating standardization of the produced data and development of secure data sharing platforms. Subsequently, sophisticated AI-based analysis methods can be used on different data sources that are being collected along the continuum of early development, from newly emerging preconception carrier testing (Sallevelt et al., 2021) to preimplantation and prenatal testing. AI can then avoid potential human errors and shorten long waiting lists, e.g. for PGT. The use of machine learning (ML), which is a branch of AI, in medicine offers an exciting prospect for disease diagnosis, monitoring and therapy. ML algorithms range from simple methods, e.g. regression and clustering, to more sophisticated approaches, e.g. artificial neural networks and deep learning. ML algorithms can be categorized into supervised or unsupervised learning. Classification is a supervised learning approach which requires labeled data, e.g. classification of PGT or NIPT results that are well annotated by specialists previously. While unsupervised learning primarily looks for patterns, e.g. clustering. The real-life example that makes use of ML in medicine more prominent is semi-supervised learning, where the clinical data are partly labeled. Semi-supervised ML systems would save enormous time and energy. ML systems that are based on multiple data sources (e.g. genomic and radiomic data) can be trained *via* deep learning. Deep learning is an approach that builds upon a cascade of several (i.e. deep) complex information layers to obtain prediction or classification models. Each layer uses the output of the preceding layer as its input, before applying different transformations to the input (Eraslan et al., 2019). These cascades of data processing are trained with labeled test data to optimize (hyper-) parameters of the model, eventually leading to the most accurate model possible (Eraslan et al., 2019). Well-trained deep-learning algorithms could, for instance, be used to identify very low abundance genetically aberrant cells and link them to their tissue of origin using WGS of cfDNA (Wan et al., 2018). The process of embryo ranking during IVF treatment is another potential application that could benefit from these techniques. However, more data collection, technology development and validation are required before robust, diagnostically valuable techniques can be brought from bench to bedside (Topol, 2019). Nonetheless, it is clear that AI has the potential to enhance the decision-making of healthcare professionals by allowing them to harness the power of vast data resources generated and stored by all healthcare systems.

Ethical exploration

Ethical aspects of applying liquid biopsy in the context of (reproductive) screening, where screening is defined as the unsolicited offer of testing to asymptomatic individuals, need to be scrutinized. Here, we discuss two criteria for sound screening, namely proportionality and respect for autonomy (Netherlands Health Council, 2008). The principle of proportionality requires that the possible benefits of screening clearly outweigh any potential risks. The autonomy requirement underlines the importance of informed and voluntary consent. The proportionality and autonomy requirements raise complex issues in the context of different types of reproductive screening, including the rapidly evolving NIPT and PGT procedures.

Given that the aim of NIPT is to facilitate well-informed, personal decision making of prospective parents about possible serious reproductive risks, the question is how to achieve this. This challenge is compounded by the prospect of whole fetal genome sequencing and analysis that could easily result in information overload. Furthermore, such broad-scope prenatal screening could violate future children's right to informational self-determination. Moreover, the morality of future NIPT-linked (research on) 'fetal personalized medicine' requires ongoing scrutiny (Dondorp et al., 2015).

In principle, non-invasive PGT to predict embryo viability would be welcome. However, as stressed in comments critical toward PGT (Mastenbroek and Repping, 2014), an important prerequisite for introducing a new test is a strong evidence base demonstrating its effectiveness and reliability. Difficult normative issues could arise if non-invasive

Table II The clinical utilities of liquid biopsy in reproductive genetics.

Clinical utility	Component	Origin	Biomarker function	Technique	Target	References
NIPT-A			Prediction of fetal trisomy 21			(Lo *et al.*, 1999)
NIPT-A			Prenatal detection of pathogenic CNVs in alpha thalassemia			(Ge *et al.*, 2013)
NIPT-A			Detection of fetal chromosomal aneuploidy			(Lo *et al.*, 2007b)
NIPT-A			Detection of fetal chromosomal aneuploidy			(Lo *et al.*, 2007a)
NIPT-M			Prenatal detection of pathogenic mutations			(Yin *et al.*, 2018)
NIPT-M			Prenatal diagnosis of monogenic diseases			(Lv *et al.*, 2015)
NIPT-M			Prenatal diagnosis of monogenic diseases			(Lam *et al.*, 2012)
NIPT-M			Prenatal diagnosis of congenital adrenal hyperplasia			(New *et al.*, 2014)
PGT-M			Preimplantation diagnosis of a-thalassemia			(Wu *et al.*, 2015)
PGT-A/M			Preimplantation diagnosis of aneuploidy and beta thalassemia			(Liu *et al.*, 2017)
Embryo quality			Prediction of embryo quality			(Scalici *et al.*, 2014)
Embryo quality			Prediction of implantation rate			(Stigliani *et al.*, 2014)
Male infertility			Identification the presence of germ cells or complete obstruction in azoospermia			(Li *et al.*, 2012)
Male infertility			Assessing diseases of semen secreting organs			(Li *et al.*, 2009)
Male infertility			Prediction of successful testicular sperm retrieval in non-obstructive azoospermia patients			(Wu *et al.*, 2013)
Fetal neurodevelopment			Non-invasive prenatal diagnosis of fetal central nervous system insult			(Goetzl *et al.*, 2019)
	cfDNA	Blood		qPCR	Quantification	
	cfRNA	Semen		NGS	Methylation	
	cf-mtDNA	ff		ddPCR	Expression profile	
	Cells	Medium		array	SNP detect/quantify	
	Exosomes			PCR	Amplicon detect/quantify	
				ELISA		

cf-mtDNA, cell-free mitochondrial DNA; ddPCR, droplet digital PCR; ELISA, enzyme-linked immuno sorbent assay; ff, follicular fluid; NGS, next-generation sequencing; NIPT-A, non-invasive prenatal testing for aneuploidies; NIPT-M, non-invasive testing for monogenic disorders; PCR, polymerase chain reaction; PGT, prenatal genetic testing; qPCR, quantitative polymerase chain reaction; SNP, single-nucleotide polymorphism.

PGT also endeavors to generate information about genetic risk factors for disorders, alone or in combination with invasive PGT methods. How then, to balance, higher/lower scores for viability and risk factors for (often complex) genetic characteristics with a lower positive predictive value? Furthermore, this may cause tension between the decision-making authority of prospective parents on one hand and of reproductive doctors on the other hand when it comes to the selection of 'the best embryo' for transfer (Wert, 2009).

Finally, even though ML may help to integrate the huge amount of data generated by multiparametric assays, the implicit morality of the algorithms involved require the development of ethics frameworks bridging AI and assisted reproduction. Clearly, the prospects of and progress in liquid biopsy-based reproductive screening require multidisciplinary research and reflection for responsible innovation.

Conclusions

Here, we described different circulating components, state-of-the-art methods to detect them, and their implications in health and disease. Specifically, we reviewed how liquid biopsy can be used to monitor patients as a whole, because the sampled components themselves are informative for their type and origin. Advanced computational methods and single-cell multi-omics will ultimately overcome some of the challenges that are associated with liquid biopsy, including the low-frequency and fragmentation of circulating components, background error rates and haplotyping phasing following NGS. AI-based interpretation of liquid biopsy profiles paves the way for individualized medicine and a much greater repertoire of non-invasive tests, which will greatly benefit patient care.

Data availability

No new data were generated in this research.

Authors' roles

G.S. and R.K.: First draft, literature search, writing, revision and display items. D.P.: Literature review, writing, revision and display items. S.J.C.S., G.W. and H.G.B.: Literature review, writing and revision. M.V.E.M, A.D.C.P., E.C., A.W., C.D.-S.: Literature review and revision. M.Z.E.: Supervision, first draft, literature search, writing and revision of all components of this manuscript.

Funding

EVA (Erfelijkheid Voortplanting & Aanleg) specialty program (grant no. KP111513) of Maastricht University Medical Centre (MUMC+) and the Horizon 2020 innovation (ERIN; grant no. EU952516) of the European Commission to M.Z.E.

Conflict of interest

M.Z.E. is co-inventor on patent applications: ZL910050-PCT/EP2011/060211-WO/2011/157846 Methods for haplotyping single cells' and ZL913096-PCT/EP2014/068315-WO/2015/028576 'Haplotyping and copy-number typing using polymorphic variant allelic frequencies'.

References

Abrahams VM, Straszewski-Chavez SL, Guller S, Mor G. First trimester trophoblast cells secrete Fas ligand which induces immune cell apoptosis. *Mol Hum Reprod* 2004;**10**:55–63. doi:10.1093/molehr/gah006

Adams DL, Stefansson S, Haudenschild C, Martin SS, Charpentier M, Chumsri S, Cristofanilli M, Tang C-M, Alpaugh RK. Cytometric characterization of circulating tumor cells captured by microfiltration and their correlation to the CellSearch((R)) CTC test. *Cytometry A* 2015;**87**:137–144.

Alberry M, Maddocks D, Jones M, Abdel Hadi M, Abdel-Fattah S, Avent N, Soothill PW. Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. *Prenat Diagn* 2007;**27**:415–418.

Ashworth TR. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. *Aust Med J* 1869;**14**:146.

Assou S, Ait-Ahmed O, El Messaoudi S, Thierry AR, Hamamah S. Non-invasive pre-implantation genetic diagnosis of X-linked disorders. *Med Hypotheses* 2014;**83**:506–508.

Assou S, Haouzi D, Mahmoud K, Aouacheria A, Guillemin Y, Pantesco V, Rème T, Dechaud H, De Vos J, Hamamah S. A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. *Mol Hum Reprod* 2008;**14**:711–719.

Awamleh Z, Gloor GB, Han VKM. Placental microRNAs in pregnancies with early onset intrauterine growth restriction and pre-eclampsia: potential impact on gene expression and pathophysiology. *BMC Med Genomics* 2019;**12**:91.

Backenroth D, Zahdeh F, Kling Y, Peretz A, Rosen T, Kort D, Zeligson S, Dror T, Kirshberg S, Burak E *et al.* Haploseek: a 24-hour all-in-one method for preimplantation genetic diagnosis (PGD) of monogenic disease and aneuploidy. *Genet Med* 2019;**21**:1390–1399.

Bayindir B, Dehaspe L, Brison N, Brady P, Ardui S, Kammoun M, Van der Veken L, Lichtenbelt K, Van den Bogaert K, Van Houdt J *et al.* Noninvasive prenatal testing using a novel analysis pipeline to screen for all autosomal fetal aneuploidies improves pregnancy management. *Eur J Hum Genet* 2015;**23**:1286–1293.

Beck J, Urnovitz HB, Riggert J, Clerici M, SchüTz E. Profile of the circulating DNA in apparently healthy individuals. *Clin Chem* 2009;**55**:730–738.

Bianchi DW. Unusual prenatal genomic results provide proof-of-principle of the liquid biopsy for cancer screening. *Clin Chem* 2018;**64**:254–256.

Bianchi DW, Chudova D, Sehnert AJ, Bhatt S, Murray K, Prosen TL, Garber JE, Wilkins-Haug L, Vora NL, Warsof S *et al.* Noninvasive prenatal testing and incidental detection of occult maternal malignancies. *JAMA* 2015;**314**:162–169.

Bianchi DW, Rava RP, Sehnert AJ. DNA sequencing versus standard prenatal aneuploidy screening. *N Engl J Med* 2014;**371**:578.

Bonucci E. Fine structure of early cartilage calcification. *J Ultrastruct Res* 1967;**20**:33–50.

Breman AM, Chow JC, U'Ren L, Normand EA, Qdaisat S, Zhao L, Henke DM, Chen R, Shaw CA, Jackson L *et al.* Evidence for feasibility of fetal trophoblastic cell-based noninvasive prenatal testing. *Prenat Diagn* 2016;**36**:1009–1019.

Bruch JF, Metezeau P, Garcia-Fonknechten N, Richard Y, Tricottet V, Hsi BL, Kitzis A, Julien C, Papiernik E. Trophoblast-like cells sorted from peripheral maternal blood using flow cytometry: a multiparametric study involving transmission electron microscopy and fetal DNA amplification. *Prenat Diagn* 1991;**11**:787–798.

Burnham P, Khush K, De Vlaminck I. Myriad applications of circulating cell-free DNA in precision organ transplant monitoring. *Ann Am Thorac Soc* 2017;**14**:S237–S241.

Butler TM, Johnson-Camacho K, Peto M, Wang NJ, Macey TA, Korkola JE, Koppie TM, Corless CL, Gray JW, Spellman PT. Exome sequencing of cell-free DNA from metastatic cancer patients identifies clinically actionable mutations distinct from primary disease. *PLoS One* 2015;**10**:e0136407.

Byron SA, Van Keuren-Jensen KR, Engelthaler DM, Carpten JD, Craig DW. Translating RNA sequencing into clinical diagnostics: opportunities and challenges. *Nat Rev Genet* 2016;**17**:257–271.

Cabello-Pinedo S, Abdulla HAN, Seth-Smith ML, Escriba M, Crespo J, Munne S, Horcajadas JA. A novel non-invasive metabolomics approach to screen embryos for aneuploidy. *Fertil Steril* 2020;**114**:e5–e6.

Chappell L, Russell AJC, Voet T. Single-cell (multi)omics technologies. *Annu Rev Genomics Hum Genet* 2018;**19**:15–41.

Che H, Villela D, Dimitriadou E, Melotte C, Brison N, Neofytou M, Van Den Bogaert K, Tsuiko O, Devriendt K, Legius E *et al.*

Noninvasive prenatal diagnosis by genome-wide haplotyping of cell-free plasma DNA. *Genet Med* 2020;**22**:962–973.

Chen SU, Lee TH, Lien YR, Tsai YY, Chang LJ, Yang YS. Microsuction of blastocoelic fluid before vitrification increased survival and pregnancy of mouse expanded blastocysts, but pretreatment with the cytoskeletal stabilizer did not increase blastocyst survival. *Fertil Steril* 2005;**84 Suppl 2**:1156–1162.

Chen WW, Balaj L, Liau LM, Samuels ML, Kotsopoulos SK, Maguire CA, Loguidice L, Soto H, Garrett M, Zhu LD *et al.* BEAMing and droplet digital PCR analysis of mutant IDH1 mRNA in glioma patient serum and cerebrospinal fluid extracellular vesicles. *Mol Ther Nucleic Acids* 2013;**2**:e109.

Chim SS, Shing TK, Hung EC, Leung TY, Lau TK, Chiu RW, Lo YM. Detection and characterization of placental microRNAs in maternal plasma. *Clin Chem* 2008;**54**:482–490.

Chim SSC, Tong YK, Chiu RWK, Lau TK, Leung TN, Chan LYS, Oudejans CBM, Ding C, Lo YMD. Detection of the placental epigenetic signature of the *maspin* gene in maternal plasma. *Proc Natl Acad Sci USA* 2005;**102**:14753–14758.

Clark SJ, Argelaguet R, Kapourani C-A, Stubbs TM, Lee HJ, Alda-Catalinas C, Krueger F, Sanguinetti G, Kelsey G, Marioni JC *et al.* scNMT-seq enables joint profiling of chromatin accessibility DNA methylation and transcription in single cells. *Nat Commun* 2018;**9**:781.

Dey SS, Kester L, Spanjaard B, Bienko M, van Oudenaarden A. Integrated genome and transcriptome sequencing of the same cell. *Nat Biotechnol* 2015;**33**:285–289.

Dharajiya NG, Grosu DS, Farkas DH, McCullough RM, Almasri E, Sun Y, Kim SK, Jensen TJ, Saldivar J-S, Topol EJ *et al.* Incidental detection of maternal neoplasia in noninvasive prenatal testing. *Clin Chem* 2018;**64**:329–335.

Dondorp W, de Wert G, Bombard Y, Bianchi DW, Bergmann C, Borry P, Chitty LS, Fellmann F, Forzano F, Hall A *et al.*; American Society of Human Genetics. Non-invasive prenatal testing for aneuploidy and beyond: challenges of responsible innovation in prenatal screening. *Eur J Hum Genet* 2015;**23**:1438–1450.

Eraslan G, Avsec Ž, Gagneur J, Theis FJ. Deep learning: new computational modelling techniques for genomics. *Nat Rev Genet* 2019;**20**:389–403.

Fan HC, Gu W, Wang J, Blumenfeld YJ, El-Sayed YY, Quake SR. Non-invasive prenatal measurement of the fetal genome. *Nature* 2012;**487**:320–324.

Feichtinger M, Vaccari E, Carli L, Wallner E, Mädel U, Figl K, Palini S, Feichtinger W. Non-invasive preimplantation genetic screening using array comparative genomic hybridization on spent culture media: a proof-of-concept pilot study. *Reprod Biomed Online* 2017;**34**:583–589.

Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DWY, Kaper F, Dawson S-J, Piskorz AM, Jimenez-Linan M, Bentley D *et al.* Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci Transl Med* 2012;**4**:136ra68.

Freidin MB, Tay A, Freydina DV, Chudasama D, Nicholson AG, Rice A, Anikin V, Lim E. An assessment of diagnostic performance of a filter-based antibody-independent peripheral blood circulating tumour cell capture paired with cytomorphologic criteria for the diagnosis of cancer. *Lung Cancer* 2014;**85**:182–185.

Galluzzi L, Palini S, Stefani SD, Andreoni F, Primiterra M, Diotallevi A, Bulletti C, Magnani M. Extracellular embryo genomic DNA and its potential for genotyping applications. *Future Sci OA* 2015;**1**:Fso62.

Ge H, Huang X, Li X, Chen S, Zheng J, Jiang H, Zhang C, Pan X, Guo J, Chen F *et al.* Noninvasive prenatal detection for pathogenic CNVs: the application in alpha-thalassemia. *PLoS One* 2013;**8**:e67464.

Gerlinger M, Rowan AJ, Horswell S, Math M, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A *et al.* Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* 2012;**366**:883–892.

Gianaroli L, Magli MC, Pomante A, Crivello AM, Cafueri G, Valerio M, Ferraretti AP. Blastocentesis: a source of DNA for preimplantation genetic testing. Results from a pilot study. *Fertil Steril* 2014;**102**:1692–1699.e6.

Giles ME, Murphy L, Krstic N, Sullivan C, Hashmi SS, Stevens B. Prenatal cfDNA screening results indicative of maternal neoplasm: survey of current practice and management needs. *Prenat Diagn* 2017;**37**:126–132.

Goetzl L, Darbinian N, Goetzl EJ. Novel window on early human neurodevelopment via fetal exosomes in maternal blood. *Ann Clin Transl Neurol* 2016;**3**:381–385.

Goetzl L, Darbinian N, Merabova N. Noninvasive assessment of fetal central nervous system insult: potential application to prenatal diagnosis. *Prenatal Diagnosis* 2019;**39**:609–615.

Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. *Nat Rev Genet* 2016;**17**:333–351.

Gu C, Liu S, Wu Q, Zhang L, Guo F. Integrative single-cell analysis of transcriptome, DNA methylome and chromatin accessibility in mouse oocytes. *Cell Res* 2019;**29**:110–123.

Hammond ER, McGillivray BC, Wicker SM, Peek JC, Shelling AN, Stone P, Chamley LW, Cree LM. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. *Fertil Steril* 2017;**107**:220–228. e225.

Handyside AH, Harton GL, Mariani B, Thornhill AR, Affara N, Shaw MA, Griffin DK. Karyomapping: a universal method for genome wide analysis of genetic disease based on mapping crossovers between parental haplotypes. *J Med Genet* 2010;**47**:651–658.

Handyside AH, Kontogianni EH, Hardy K, Winston RM. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature* 1990;**344**:768–770.

Hatt L, Brinch M, Singh R, Møller K, Lauridsen RH, Schlütter JM, Uldbjerg N, Christensen B, Kølvraa S. A new marker set that identifies fetal cells in maternal circulation with high specificity. *Prenat Diagn* 2014;**34**:1066–1072.

Ho JR, Arrach N, Rhodes-Long K, Ahmady A, Ingles S, Chung K, Bendikson KA, Paulson RJ, McGinnis LK. Pushing the limits of detection: investigation of cell-free DNA for aneuploidy screening in embryos. *Fertil Steril* 2018;**110**:467–475.e2.

Holdenrieder S, Stieber P, Bodenmüller H, Busch M, Von Pawel J, Schalhorn A, Nagel D, Seidel D. Circulating nucleosomes in serum. *Ann N Y Acad Sci* 2001;**945**:93–102.

Hong Y, Wang C, Fu Z, Liang H, Zhang S, Lu M, Sun W, Ye C, Zhang C-Y, Zen K *et al.* Systematic characterization of seminal

plasma piRNAs as molecular biomarkers for male infertility. *Sci Rep* 2016;**6**:24229.

Hou Y, Fan W, Yan L, Li R, Lian Y, Huang J, Li J, Xu L, Tang F, Xie XS *et al*. Genome analyses of single human oocytes. *Cell* 2013; **155**:1492–1506.

Huang S, Li H, Ding X, Xiong C. Presence and characterization of cell-free seminal RNA in healthy individuals: implications for noninvasive disease diagnosis and gene expression studies of the male reproductive system. *Clin Chem* 2009;**55**:1967–1976.

Huang J, Yan L, Fan W, Zhao N, Zhang Y, Tang F, Xie XS, Qiao J. Validation of multiple annealing and looping-based amplification cycle sequencing for 24-chromosome aneuploidy screening of cleavage-stage embryos. *Fertil Steril* 2014;**102**:1685–1691.

Huang L, Ma F, Chapman A, Lu S, Xie XS. Single-cell whole-genome amplification and sequencing: methodology and applications. *Annu Rev Genomics Hum Genet* 2015;**16**:79–102.

Huang X, Yuan T, Tschannen M, Sun Z, Jacob H, Du M, Liang M, Dittmar RL, Liu Y, Liang M *et al*. Characterization of human plasma-derived exosomal RNAs by deep sequencing. *BMC Genomics* 2013;**14**:319.

Jensen TJ, Kim SK, Zhu Z, Chin C, Gebhard C, Lu T, Deciu C, van den Boom D, Ehrich M. Whole genome bisulfite sequencing of cell-free DNA and its cellular contributors uncovers placenta hypomethylated domains. *Genome Biol* 2015;**16**:78–78.

Ji X, Chen F, Zhou Y, Li J, Yuan Y, Mo Y, Liu Q, Tseng J-Y, Shih-Chieh Lin D, Shen S-H *et al*. Copy number variation profile in non-invasive prenatal testing (NIPT) can identify co-existing maternal malignancies: Case reports and a literature review. *Taiwan J Obstet Gynecol* 2018;**57**:871–877.

Kalousek DK, Vekemans M. Confined placental mosaicism. *J Med Genet* 1996;**33**:529–533.

Kansaku K, Munakata Y, Itami N, Shirasuna K, Kuwayama T, Iwata H. Mitochondrial dysfunction in cumulus-oocyte complexes increases cell-free mitochondrial DNA. *J Reprod Dev* 2018;**64**: 261–266.

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics. *Br J Cancer* 1972;**26**:239–257.

Khier S, Lohan L. Kinetics of circulating cell-free DNA for biomedical applications: critical appraisal of the literature. *Future Sci Oa* 2018; **4**:FSO295.

Ko J, Carpenter E, Issadore D. Detection and isolation of circulating exosomes and microvesicles for cancer monitoring and diagnostics using micro-/nano-based devices. *Analyst* 2016;**141**:450–460.

Kølvraa S, Singh R, Normand EA, Qdaisat S, van den Veyver IB, Jackson L, Hatt L, Schelde P, Uldbjerg N, Vestergaard EM *et al*. Genome-wide copy number analysis on DNA from fetal cells isolated from the blood of pregnant women. *Prenat Diagn* 2016;**36**: 1127–1134.

Kotsopoulou I, Tsoplou P, Mavrommatis K, Kroupis C. Non-invasive prenatal testing (NIPT): limitations on the way to become diagnosis. *Diagnosis (Berl)* 2015;**2**:141–158.

Krabchi K, Gros-Louis F, Yan J, Bronsard M, Massé J, Forest J-C, Drouin R. Quantification of all fetal nucleated cells in maternal blood between the 18th and 22nd weeks of pregnancy using molecular cytogenetic techniques. *Clin Genet* 2001;**60**:145–150.

Kuliev A, Rechitsky S. Preimplantation genetic testing: current challenges and future prospects. *Expert Rev Mol Diagn* 2017;**17**: 1071–1088.

Kumarswamy R, Bauters C, Volkmann I, Maury F, Fetisch J, Holzmann A, Lemesle G, de Groote P, Pinet F, Thum T. Circulating long noncoding RNA, LIPCAR, predicts survival in patients with heart failure. *Circ Res* 2014;**114**:1569–1575.

Kuznyetsov V, Madjunkova S, Abramov R, Antes R, Ibarrientos Z, Motamedi G, Zaman A, Kuznyetsova I, Librach CL. Minimally invasive cell-free human embryo aneuploidy testing (miPGT-A) utilizing combined spent embryo culture medium and blastocoel fluid—towards development of a clinical assay. *Sci Rep* 2020;**10**:7244.

Laird PW. Principles and challenges of genome-wide DNA methylation analysis. *Nat Rev Genet* 2010;**11**:191–203.

Lam KW, Jiang P, Liao GJ, Chan KC, Leung TY, Chiu RW, Lo YM. Noninvasive prenatal diagnosis of monogenic diseases by targeted massively parallel sequencing of maternal plasma: application to beta-thalassemia. *Clin Chem* 2012;**58**:1467–1475.

Lane M, Zander-Fox DL, Hamilton H, Jasper MJ, Hodgson BL, Fraser M, Bell F. Ability to detect aneuploidy from cell free DNA collected from media is dependent on the stage of development of the embryo. *Fertil Steril* 2017;**108**:e61.

Leary RJ, Sausen M, Kinde I, Papadopoulos N, Carpten JD, Craig D, O'Shaughnessy J, Kinzler KW, Parmigiani G, Vogelstein B *et al*. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. *Sci Transl Med* 2012;**4**: 162ra154.

Lee TH, Montalvo L, Chrebtow V, Busch MP. Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. *Transfusion* 2001; **41**:276–282.

Lenaerts L, Brison N, Maggen C, Vancoillie L, Che H, Vandenberghe P, Dierickx D, Michaux L, Dewaele B, Neven P *et al*. Comprehensive genome-wide analysis of routine non-invasive test data allows cancer prediction: a single-center retrospective analysis of over 85,000 pregnancies. *EClinicalMedicine* 2021;**35**:100856.

Li H, Wu C, Gu X, Xiong C. A novel application of cell-free seminal mRNA: non-invasive identification of the presence of germ cells or complete obstruction in men with azoospermia. *Hum Reprod* 2012;**27**:991–997.

Li HG, Huang SY, Zhou H, Liao AH, Xiong CL. Quick recovery and characterization of cell-free DNA in seminal plasma of normozoospermia and azoospermia: implications for non-invasive genetic utilities. *Asian J Androl* 2009;**11**:703–709.

Li L, Guo F, Gao Y, Ren Y, Yuan P, Yan L, Li R, Lian Y, Li J, Hu B *et al*. Single-cell multi-omics sequencing of human early embryos. *Nat Cell Biol* 2018;**20**:847–858.

Liu W, Liu J, Du H, Ling J, Sun X, Chen D. Non-invasive preimplantation aneuploidy screening and diagnosis of beta thalassemia IVSII654 mutation using spent embryo culture medium. *Ann Med* 2017;**49**:319–328.

Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;**350**:485–487.

Lo YM, Lau TK, Zhang J, Leung TN, Chang AM, Hjelm NM, Elmes RS, Bianchi DW. Increased fetal DNA concentrations in the

plasma of pregnant women carrying fetuses with trisomy 21. *Clin Chem* 1999;**45**:1747–1751.

Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, Wainscoat JS, Johnson PJ, Chang AM, Hjelm NM. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998;**62**: 768–775.

Lo YMD, Chan KCA, Sun H, Chen EZ, Jiang P, Lun FMF, Zheng YW, Leung TY, Lau TK, Cantor CR *et al*. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci Transl Med* 2010;**2**:61ra91.

Lo YMD, Lun FMF, Chan KCA, Tsui NBY, Chong KC, Lau TK, Leung TY, Zee BCY, Cantor CR, Chiu RWK. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. *Proc Natl Acad Sci U S A* 2007a;**104**:13116–13121.

Lo YMD, Tsui NBY, Chiu RWK, Lau TK, Leung TN, Heung MMS, Gerovassili A, Jin Y, Nicolaides KH, Cantor CR *et al*. Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. *Nat Med* 2007b;**13**:218–223.

Lou DI, Hussmann JA, McBee RM, Acevedo A, Andino R, Press WH, Sawyer SL. High-throughput DNA sequencing errors are reduced by orders of magnitude using circle sequencing. *Proc Natl Acad Sci U S A* 2013;**110**:19872–19877.

Lv W, Wei X, Guo R, Liu Q, Zheng Y, Chang J, Bai T, Li H, Zhang J, Song Z *et al*. Noninvasive prenatal testing for Wilson disease by use of circulating single-molecule amplification and resequencing technology (cSMART). *Clin Chem* 2015;**61**:172–181.

Macaulay IC, Haerty W, Kumar P, Li YI, Hu TX, Teng MJ, Goolam M, Saurat N, Coupland P, Shirley LM *et al*. G&T-seq: parallel sequencing of single-cell genomes and transcriptomes. *Nat Methods* 2015;**12**:519–522.

Mandel P, Metais P. Les acides nucléiques du plasma sanguin chez l'homme. *C R Seances Soc Biol Fil* 1948;**142**:241–243.

Masset H, Zamani Esteki M, Dimitriadou E, Dreesen J, Debrock S, Derhaag J, Derks K, Destouni A, Drüsedau M, Meekels J *et al*. Multi-centre evaluation of a comprehensive preimplantation genetic test through haplotyping-by-sequencing. *Hum Reprod* 2019; **34**:1608–1619.

Mastenbroek S, Repping S. Preimplantation genetic screening: back to the future. *Hum Reprod* 2014;**29**:1846–1850.

Max KEA, Bertram K, Akat KM, Bogardus KA, Li J, Morozov P, Ben-Dov IZ, Li X, Weiss ZR, Azizian A *et al*. Human plasma and serum extracellular small RNA reference profiles and their clinical utility. *Proc Natl Acad Sci U S A* 2018;**115**:E5334–E5343.

Mazzini C, Pinzani P, Salvianti F, Scatena C, Paglierani M, Ucci F, Pazzagli M, Massi D. Circulating tumor cells detection and counting in uveal melanomas by a filtration-based method. *Cancers (Basel)* 2014;**6**:323–332.

Meng W, He C, Hao Y, Wang L, Li L, Zhu G. Prospects and challenges of extracellular vesicle-based drug delivery system: considering cell source. *Drug Deliv* 2020;**27**:585–598.

Mersy E, Faas BHW, Spierts S, Houben LMH, Macville MVE, Frints SGM, Paulussen ADC, Veltman JA. Cell-free RNA is a reliable fetoplacental marker in noninvasive fetal sex determination. *Clin Chem* 2015;**61**:1515–1523.

Natesan SA, Bladon AJ, Coskun S, Qubbaj W, Prates R, Munne S, Coonen E, Dreesen JCFM, Stevens SJC, Paulussen ADC *et al*. Genome-wide karyomapping accurately identifies the inheritance of single-gene defects in human preimplantation embryos *in vitro*. *Genet Med* 2014;**16**:838–845.

Navin NE. The first five years of single-cell cancer genomics and beyond. *Genome Res* 2015;**25**:1499–1507.

Neoh KH, Hassan AA, Chen A, Sun Y, Liu P, Xu K-F, Wong AST, Han RPS. Rethinking liquid biopsy: microfluidic assays for mobile tumor cells in human body fluids. *Biomaterials* 2018;**150**:112–124.

Netherlands Health Council. *Screening: Between Hope and Hype*. The Hague, 2008. https://www.healthcouncil.nl/documents/advisory-reports/2008/04/01/screening-between-hope-and-hype

New MI, Tong YK, Yuen T, Jiang P, Pina C, Chan KCA, Khattab A, Liao GJW, Yau M, Kim S-M *et al*. Noninvasive prenatal diagnosis of congenital adrenal hyperplasia using cell-free fetal DNA in maternal plasma. *J Clin Endocrinol Metab* 2014;**99**:E1022–E1030.

Palini S, Galluzzi L, De Stefani S, Bianchi M, Wells D, Magnani M, Bulletti C. Genomic DNA in human blastocoele fluid. *Reprod Biomed Online* 2013;**26**:603–610.

Pegtel DM, Gould SJ. Exosomes. *Annu Rev Biochem* 2019;**88**: 487–514.

Pernemalm M, Sandberg A, Zhu Y, Boekel J, Tamburro D, Schwenk JM, Björk A, Wahren-Herlenius M, Åmark H, Östenson C-G *et al*. In-depth human plasma proteome analysis captures tissue proteins and transfer of protein variants across the placenta. *Elife* 2019;**8**: e41608.

Pertl B, Sekizawa A, Samura O, Orescovic I, Rahaim PT, Bianchi DW. Detection of male and female fetal DNA in maternal plasma by multiplex fluorescent polymerase chain reaction amplification of short tandem repeats. *Hum Genet* 2000;**106**:45–49.

Poon LLM, Leung TN, Lau TK, Lo YMD. Presence of fetal RNA in maternal plasma. *Clin Chem* 2000;**46**:1832–1834.

Rabinowitz T, Polsky A, Golan D, Danilevsky A, Shapira G, Raff C, Basel-Salmon L, Matar RT, Shomron N. Bayesian-based noninvasive prenatal diagnosis of single-gene disorders. *Genome Res* 2019; **29**:428–438.

Raman L, Dheedene A, De Smet M, Van Dorpe J, Menten B. WisecondorX: improved copy number detection for routine shallow whole-genome sequencing. *Nucleic Acids Res* 2019;**47**: 1605–1614.

Russell LM, Strike P, Browne CE, Jacobs PA. X chromosome loss and ageing. *Cytogenet Genome Res* 2007;**116**:181–185.

Sallevelt SCEH, Stegmann APA, de Koning B, Velter C, Steyls A, van Esch M, Lakeman P, Yntema H, Esteki MZ, de Die-Smulders CEM *et al*. Diagnostic exome-based preconception carrier testing in consanguineous couples: results from the first 100 couples in clinical practice. *Genet Med* 2021;**23**:1125–1136.

Scalici E, Traver S, Molinari N, Mullet T, Monforte M, Vintejoux E, Hamamah S. Cell-free DNA in human follicular fluid as a biomarker of embryo quality. *Hum Reprod* 2014;**29**:2661–2669.

Schmorl G. *Pathologisch-Anatomische Untersuchungen Über Puerperal-Eklampsie*. Vogel, 1893.

Sermon K, Van Steirteghem A, Liebaers I. Preimplantation genetic diagnosis. *Lancet* 2004;**363**:1633–1641.

Shamonki MI, Jin H, Haimowitz Z, Liu L. Proof of concept: preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. *Fertil Steril* 2016;**106**:1312–1318.

Shi J, Zhang R, Li J, Zhang R. Size profile of cell-free DNA: a beacon guiding the practice and innovation of clinical testing. *Theranostics* 2020;**10**:4737–4748.

Smith J, Kean V, Bianchi DW, Feldman G, Petrucelli N, Simon M, Gonik B. Cell-free DNA results lead to unexpected diagnosis. *Clin Case Rep* 2017;**5**:1323–1326.

Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA comprises an *in vivo* nucleosome footprint that informs its tissues-of-origin. *Cell* 2016;**164**:57–68.

Stigliani S, Anserini P, Venturini PL, Scaruffi P. Mitochondrial DNA content in embryo culture medium is significantly associated with human embryo fragmentation. *Hum Reprod* 2013;**28**:2652–2660.

Stigliani S, Persico L, Lagazio C, Anserini P, Venturini PL, Scaruffi P. Mitochondrial DNA in Day 3 embryo culture medium is a novel, non-invasive biomarker of blastocyst potential and implantation outcome. *Mol Hum Reprod* 2014;**20**:1238–1246.

Straver R, Sistermans EA, Holstege H, Visser A, Oudejans CB, Reinders MJ. WISECONDOR: detection of fetal aberrations from shallow sequencing maternal plasma based on a within-sample comparison scheme. *Nucleic Acids Res* 2014;**42**:e31.

Sun K, Jiang P, Chan KCA, Wong J, Cheng YKY, Liang RHS, Chan W-K, Ma ESK, Chan SL, Cheng SH *et al.* Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. *Proc Natl Acad Sci U S A* 2015;**112**:E5503–E5512.

Tang F, Lao K, Surani MA. Development and applications of single-cell transcriptome analysis. *Nat Methods* 2011;**8**:S6–S11.

Tian T, Wang J, Zhou X. A review: microRNA detection methods. *Org Biomol Chem* 2015;**13**:2226–2238.

Tixeira R, Caruso S, Paone S, Baxter AA, Atkin-Smith GK, Hulett MD, Poon IK. Defining the morphologic features and products of cell disassembly during apoptosis. *Apoptosis* 2017;**22**:475–477.

Tjoa ML, Cindrova-Davies T, Spasic-Boskovic O, Bianchi DW, Burton GJ. Trophoblastic oxidative stress and the release of cell-free feto-placental DNA. *Am J Pathol* 2006;**169**:400–404.

Topol EJ. High-performance medicine: the convergence of human and artificial intelligence. *Nat Med* 2019;**25**:44–56.

Trams EG, Lauter CJ, Salem N Jr, Heine U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochim Biophys Acta* 1981;**645**:63–70.

van der Meij KRM, Sistermans EA, Macville MVE, Stevens SJC, Bax CJ, Bekker MN, Bilardo CM, Boon EMJ, Boter M, Diderich KEM *et al.*; Dutch NIPT Consortium. TRIDENT-2: national implementation of genome-wide non-invasive prenatal testing as a first-tier screening test in the Netherlands. *Am J Hum Genet* 2019;**105**:1091–1101.

van der Vaart M, Semenov DV, Kuligina EV, Richter VA, Pretorius PJ. Characterisation of circulating DNA by parallel tagged sequencing on the 454 platform. *Clin Chim Acta* 2009;**409**:21–27.

van Schendel RV, Page-Christiaens GCML, Beulen L, Bilardo CM, de Boer MA, Coumans ABC, Faas BHW, van Langen IM, Lichtenbelt KD, van Maarle MC *et al.*; for the Dutch NIPT Consortium. Women's experience with non-invasive prenatal testing and emotional well-being and satisfaction after test-results. *J Genet Counsel* 2017;**26**:1348–1356.

Vanneste E, Voet T, Le Caignec C, Ampe M, Konings P, Melotte C, Debrock S, Amyere M, Vikkula M, Schuit F *et al.* Chromosome instability is common in human cleavage-stage embryos. *Nat Med* 2009;**15**:577–583.

Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, Mercader A, Meseguer M, Blesa D, Moreno I *et al.* Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. *Hum Reprod* 2018;**33**:745–756.

Vermeesch JR, Voet T, Devriendt K. Prenatal and pre-implantation genetic diagnosis. *Nat Rev Genet* 2016;**17**:643–656.

Vona G, Béroud C, Benachi A, Quenette A, Bonnefont JP, Romana S, Dumez Y, Lacour B, Paterlini-Bréchot P. Enrichment, immunomorphological, and genetic characterization of fetal cells circulating in maternal blood. *Am J Pathol* 2002;**160**:51–58.

Vossaert L, Wang Q, Salman R, McCombs AK, Patel V, Qu C, Mancini MA, Edwards DP, Malovannaya A, Liu P *et al.* Validation studies for single circulating trophoblast genetic testing as a form of noninvasive prenatal diagnosis. *Am J Hum Genet* 2019;**105**:1262–1273.

Wahid B, Rafique S, Ali A, Waqar M, Nabi G, Wasim M, Idrees M. Biomarkers for diagnosis of pre-eclampsia and endometriosis. *Biomark Med* 2018;**12**:1161–1173.

Wan N, Weinberg D, Liu T-y, Niehaus K, Delubac D, Kannan A, White B, Ariazi EA, Bailey M, Bertin M *et al.* (2018). Machine learning enables detection of early-stage colorectal cancer by whole-genome sequencing of plasma cell-free DNA. *bioRxiv*, 10.1101/478065.

Wang Y, Li S, Wang W, Dong Y, Zhang M, Wang X, Yin C. Cell-free DNA screening for sex chromosome aneuploidies by non-invasive prenatal testing in maternal plasma. *Mol Cytogenet* 2020;**13**:10.

Warkiani ME, Khoo BL, Wu L, Tay AK, Bhagat AA, Han J, Lim CT. Ultra-fast, label-free isolation of circulating tumor cells from blood using spiral microfluidics. *Nat Protoc* 2016;**11**:134–148.

Wert GD. Preimplantation genetic diagnosis. In: Harper J (ed). *Preimplantation Genetic Testing: Normative Reflections*. Cambridge University Press, 2nd edn, 2009,259–273.

Wu H, Ding C, Shen X, Wang J, Li R, Cai B, Xu Y, Zhong Y, Zhou C. Medium-based noninvasive preimplantation genetic diagnosis for human alpha-thalassemias-SEA. *Medicine (Baltimore)* 2015;**94**:e669.

Wu W, Qin Y, Li Z, Dong J, Dai J, Lu C, Guo X, Zhao Y, Zhu Y, Zhang W *et al.* Genome-wide microRNA expression profiling in idiopathic non-obstructive azoospermia: significant up-regulation of miR-141, miR-429 and miR-7-1-3p. *Hum Reprod* 2013;**28**:1827–1836.

Xu J, Fang R, Chen L, Chen D, Xiao J-P, Yang W, Wang H, Song X, Ma T, Bo S *et al.* Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for *in vitro* fertilization. *Proc Natl Acad Sci U S A* 2016;**113**:11907–11912.

Yin X, Du Y, Zhang H, Wang Z, Wang J, Fu X, Cui Y, Chen C, Liang J, Xuan Z *et al.* Identification of a de novo fetal variant in osteogenesis imperfecta by targeted sequencing-based noninvasive prenatal testing. *J Hum Genet* 2018;**63**:1129–1137.

Zamani Esteki M, Dimitriadou E, Mateiu L, Melotte C, Van der Aa N, Kumar P, Das R, Theunis K, Cheng J, Legius E *et al.* Concurrent whole-genome haplotyping and copy-number profiling of single cells. *Am J Hum Genet* 2015;**96**:894–912.

Zamani Esteki M, Viltrop T, Tšuiko O, Tiirats A, Koel M, Nõukas M, Žilina O, Teearu K, Marjonen H, Kahila H *et al*. *In vitro* fertilization does not increase the incidence of de novo copy number alterations in fetal and placental lineages. *Nat Med* 2019;**25**:1699–1705.

Zhang J, Li J, Saucier JB, Feng Y, Jiang Y, Sinson J, McCombs AK, Schmitt ES, Peacock S, Chen S *et al*. Non-invasive prenatal sequencing for multiple Mendelian monogenic disorders using circulating cell-free fetal DNA. *Nat Med* 2019;**25**:439–447.

Zhu D, Zhang J, Cao S, Zhang J, Heng BC, Huang M, Ling X, Duan T, Tong GQ. Vitrified-warmed blastocyst transfer cycles yield higher pregnancy and implantation rates compared with fresh blastocyst transfer cycles—time for a new embryo transfer strategy? *Fertil Steril* 2011;**95**:1691–1695.

Zipursky A, Hull A, White FD, Israels LG. Foetal erythrocytes in the maternal circulation. *Lancet* 1959;**1**:451–452.