Innovations in Down syndrome screening

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Artwork representing the genetic development of a human foetus. The hollow inside of the foetus is being "filled" by chromosomes.

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Innovations in Down syndrome screening

Innovaties in Down syndroom screening
(met een samenvatting in het Nederlands)

Proefschrift

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door

Maria Pietronella Hendrika Koster
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Never desert your own line of talent.
Be what nature intended you for,
and you will succeed.

(Sydney Smith)

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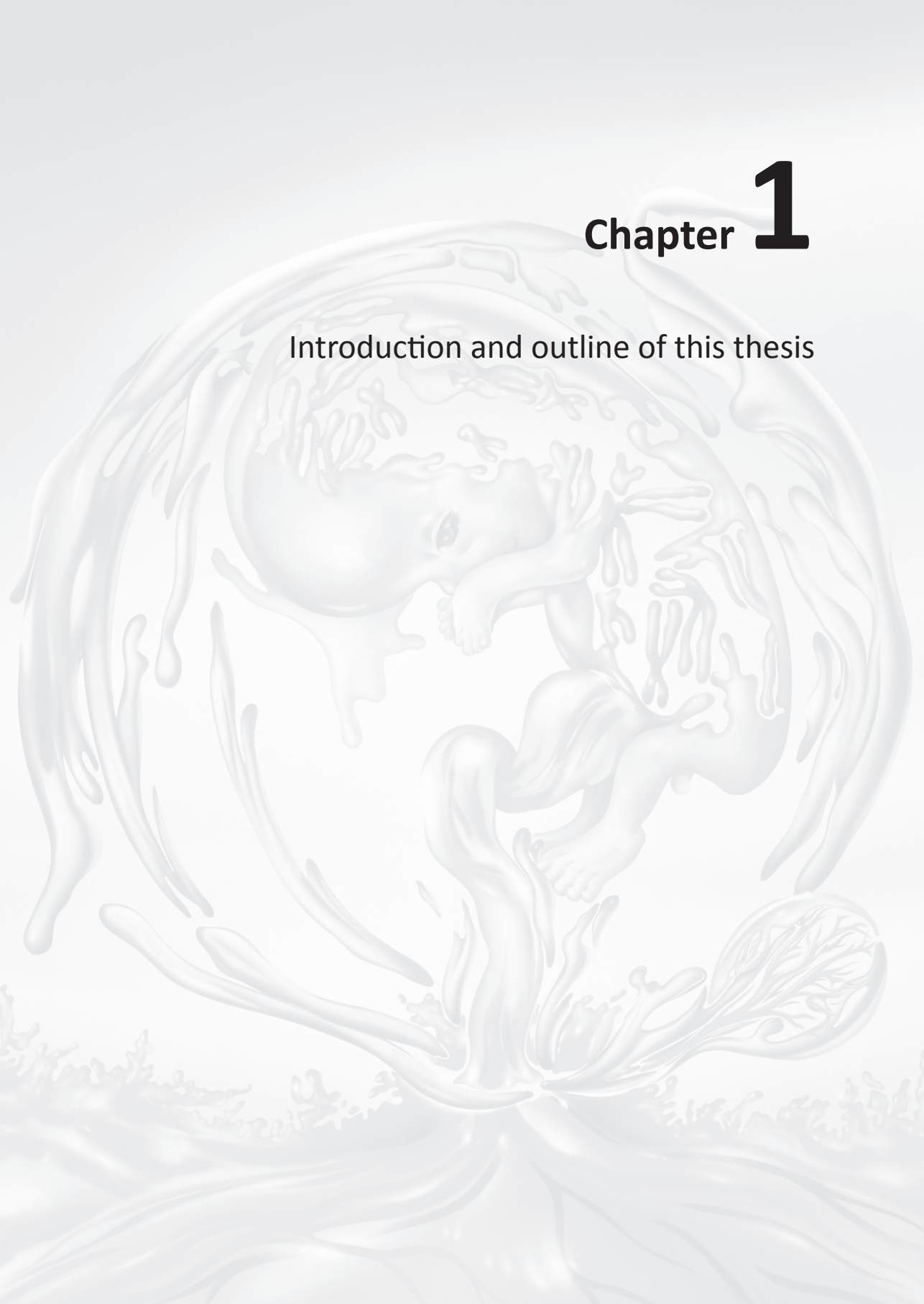
Chapter 1

Introduction and outline of this thesis



A historical perspective of Down syndrome

An accurate phenotypic description of Down syndrome (DS) was published by John Langdon Down in 1866 [1]. Following descriptions of Esquirol and Séguin [2, 3], who wrote about phenotypic differences between mentally retarded humans, Down was the first to make the distinction between the phenotype which is now called DS and other disorders. He made this distinction based on an ethnic classification in which he discerned four types; the Ethiopian type, the Malay type, the American type and the Mongolian type [1]. The latter was described by Down as: *"The face is flat and broad, and destitute of prominence. The cheeks are roundish, and extend laterally. The eyes are obliquely placed, and the internal canthi, more than normally distanced from one another. The palpebral fissure is very narrow. The forehead is wrinkled transversely from the constant assistance, which the levatores palpebrarum derive from the occipitofrontalis muscle in the opening of the eyes. The lips are large and thick with transverse fissures. The tongue is long, thick, and is much roughened. The nose is small."* Down noticed that this 'mongolism' occurred in more that 10% of all mentally retarded children and that it was always congenital.

Twenty years later, in 1886, Shuttleworth pointed out that children with DS were mostly born from older mothers and that they were often the last born child. Based on these observations he concluded that the risk of having a child with DS increased with maternal age [4].

At the end of the 19th century, the principle of inheritance was explained by the discovery of chromosomes in living organisms. In 1909, Morgan and colleagues began to study the chromosomes of Drosophila (fruit flies), which were very suitable for genetic studies because they breed quickly and only have four chromosomes. During their experiments it was, among other things, discovered that occasionally Drosophila possessed three sex chromosomes instead of two, showing a pattern of XXY or XYY, an abnormality which they called 'trisomy' [5, 6]. Since this trisomy occurred when two copies of a chromosome failed to disjoin properly, it was described as non-disjunction.

Somewhat later, in the 1930s, two researchers independently linked non-disjunction to DS. Waardenburg stated that, due to the extended clinical features of humans with DS, the syndrome might very well be caused by something as complicated as a chromosomal disorder [7]. Bleyer proposed that DS occurs with fertilization or has already occurred before, during the period of maturation of the ovum or spermatozoon [8]. Therefore, he thought that a chromosomal abnormality such as non-disjunction was most likely to cause DS.

Non-disjunction as a cause of DS, as proposed by Waardenburg and Bleyer, was called the 'mutation theory'. However, during that time there were others with opposing theories which were not linked to the Drosophila research of Morgan. The 'hereditary theory' was based on DS being a rare and pathogenic trait which could spontaneously occur [9, 10]. In the

'defective ovum theory' it was proposed that due to a diminished viability of the ovum (caused by endocrine imbalance or aging), complete failure of development occurred leading to the birth of children with DS [11-13].

Finally, in 1959, a few years after it had been established that human tissues normally contain 46 chromosomes, Lejeune and Jacobs independently discovered the presence of an extra chromosome in children with DS [14, 15]. Lejeune suggested, principally based on the Drosophila research, that the presence of an extra chromosome could well be explained in terms of non-disjunction. As individual chromosomes were identified, it appeared that the extra chromosome in DS was always the 21st chromosome. Therefore, DS was since then referred to as trisomy 21.

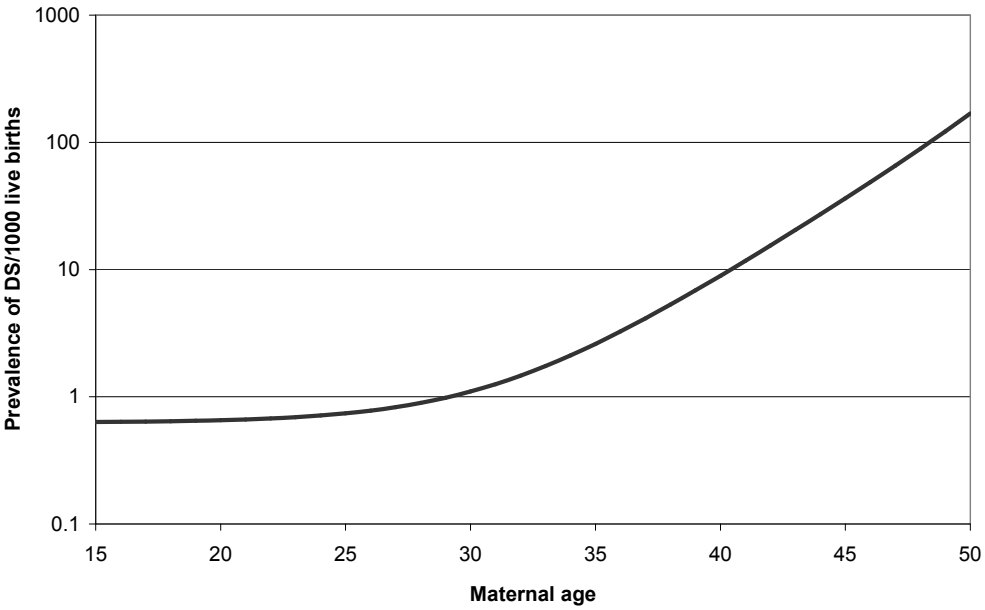


Figure 1 – The relationship between maternal age and the risk of having a child with Down syndrome based on data from Cuckle et al. [16].

Prenatal screening for Down syndrome

In 1966 the first chromosome analysis of amniotic fluid was performed [17]. This development allowed for the prenatal detection of DS, which was first achieved in 1968 [18]. The relationship between the risk of having a child with DS and advanced maternal age had been known for a long time [4, 19]. A statistical estimation of this relationship is shown in *Figure 1*. Because of an increased risk of DS, in many countries women above a certain age (usually above 35-38 years) were offered prenatal diagnosis by means of amniocentesis.

In 1972 it was discovered that very high levels of alpha fetoprotein (AFP) were present in the amniotic fluid of women carrying a child with a neural tube defect (NTD) [20]. Two years later the association between high AFP levels and NTD was also seen in second trimester maternal serum samples [21, 22], allowing for a non-invasive screening method for NTD [23]. Again a few years later, in 1984, it was found that, opposite to the high AFP levels in NTD pregnancies, decreased maternal serum levels of AFP in the second trimester of pregnancy could be linked to DS [24, 25]. This meant that prenatal screening for NTD could be extended with the screening for DS. This way, women of advanced maternal age could now be offered a screening test before opting for an invasive amniocentesis that bears a certain risk of miscarriage [26, 27]. In the Netherlands, screening for NTD using AFP as a marker was first performed in 1977 [28] and, subsequently, screening for DS was adopted in 1988 [29].

The discovery of AFP as a second trimester screening marker for DS triggered researchers to look for other potential screening markers to even further improve the prenatal detection by screening. In 1987, two new screening markers were presented. Maternal serum levels of human chorion gonadotropin (hCG) were shown to be, on average, higher in DS pregnancies [30] while levels of unconjugated estriol (uE_3) were mostly decreased in DS [31]. A year later, Wald and colleagues reported on a new method of screening using the three biochemical markers (AFP, hCG and uE_3) together with maternal age as parameters in a single test [32]. This test became known as the 'triple test'. With the triple test 60% of all Down syndrome cases could be prenatally detected at a 5% false positive rate (FPR) [32], which was a significant improvement compared to the detection of the previous screening method based on maternal age and AFP only [24]. The triple test became increasingly popular as a screening test for DS and started to be carried out routinely in several countries. The most optimal cut-off risk for the screening was calculated to be 1 in 250 [33]. During the early 1990s, the triple test was adjusted by the replacement of hCG with the free beta subunit of hCG (fβ-hCG) [34, 35]. Moreover, in 1996, inhibin-A was found to contribute to the current triple test [36] and with the addition of inhibin-A the 'quadruple test' was conceived.

In the Netherlands screening for DS with the triple test has been carried out since 1991 [29]. By then there was not yet an official prenatal screening programme, but every pregnant woman, despite her age, could opt for a triple test. This changed in 1996, when the Population Screening Act became valid. Under this act screening for DS would acquire a license. In the absence of such a license actively offering prenatal screening by a health professional became illegal. However, it was still allowed to carry out the triple test if pregnant women specifically requested it. This development led to a decline in the number of triple tests carried out in the Netherlands (*Figure 2*).

In the meantime the focus of prenatal screening for DS shifted more towards the first-trimester of pregnancy. This development was in part due to the applicability of chorionic villus sampling, a technique that allows for karyotyping already in the first-trimester. Thus,

it became possible to detect DS earlier in pregnancy, which subsequently allowed for earlier termination of pregnancy. On the other hand, first-trimester screening would not include screening for NTD. However, advanced ultrasound techniques were developed promising high detection rates for NTD in the second trimester.

Except for fβ-hCG [37], the parameters in the current triple test did not perform well in the distinction between DS and euploid pregnancies in the first trimester. So, to come up with a proper test, new first-trimester screening markers were necessary. In 1991, it was found that maternal serum pregnancy-associated plasma protein A (PAPP-A) was reduced twice in DS pregnancies [38]. Besides PAPP-A, more potential markers were studied (e.g. SP1 [39] and CA125 [40]), but none of those turned out to be worth adding to the screening test. The search for DS screening markers was not limited to biochemical markers; an enlarged nuchal translucency (NT) on a first-trimester ultrasound scan also turned out to be predictive for DS [41, 42]. Combining these three screening markers (fβ-hCG, PAPP-A and NT) with maternal age, using a risk calculation method similar to that of the triple test, originated the 'first-trimester combined test' [43]. Over the years, several studies have been published showing that with the first-trimester combined test approximately 85-90% of all DS cases could be detected at a 5% FPR [44-48].

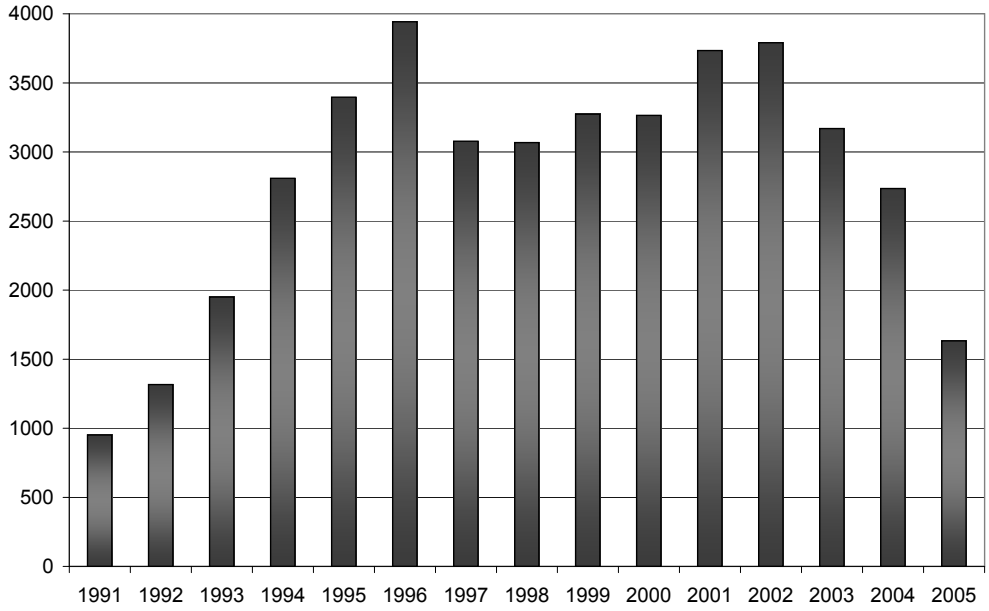


Figure 2 – Number of triple test requests (n = 42,112) in the Netherlands per annum over 1991–2005. In 1996 the Population screening act came into force and therefore the number of triple tests dropped in 1997. Since 2002 the first-trimester combined test has been carried out in the Netherlands. This resulted in a steady decline of the number of triple tests, with currently almost insignificant numbers. Based on data from Wortelboer et al. [49].

The first-trimester combined test was carried out in the Netherlands from 2002 onwards. This resulted in a steady decline of the number of triple tests, with currently almost insignificant numbers (*Figure 2*). Still, it was only allowed to offer a pregnant woman information on a risk estimation test for DS on her explicit request. In 2001 and 2004 two reports of the Dutch Health Council (the major advisory body to the Ministry of Health, Welfare and Sports) tried to combine the demands of the Population Screening Act with the needs of pregnant women and health professionals [50, 51]. The Health Council advised to allow informing all pregnant women on the possibility of having a screening test for DS with the first-trimester combined test as the test of choice and, thereupon, this advice was adopted by the ministry [52]. Under strict guidelines issued by the Centre for Population Research the screening policy for DS was fully implemented as of January 1, 2007.

Aims and outline of this thesis

In recent years several studies have been performed to evaluate the performance of the first-trimester combined test in the Netherlands [53, 54]. The detection of DS, which is around 75%, appears to be lower than reported in other countries [48, 55, 56]. This indicates that, at least in the Netherlands, there is a need for improvement of first-trimester DS screening.

The aims of this thesis are:
1) To study the quality of the current first-trimester DS screening programme and to provide ways of improvement
2) To study the applicability of current and potential biochemical screening markers for DS, trisomy 18 and trisomy 13 in singleton and twin pregnancies
3) To identify new biochemical screening markers for DS using proteomics techniques

An extended review of the literature to study the physiology of normal placental development versus placental development in DS pregnancies is given in **chapter 2**.
Several lacunas in the current screening programme are discussed. This includes the accuracy of the determination of gestational age for prenatal screening (**chapter 3**) and the quality of NT measurements (**chapter 4**). Moreover, the most optimal moment of testing (early or later in the first trimester) was evaluated to obtain the highest possible detection rates (**chapter 5**). In **chapter 6, 7, 8 and 9** the possible application of current, but also potential new prenatal screening markers is described. Besides DS pregnancies, the applicability of several biochemical markers was evaluated in other trisomic pregnancies and in twin pregnancies. The last part of this thesis describes the discovery and validation of completely new biochemical screening markers for DS using proteomics. Proteomics is the study of proteins with regard to their structure, functional characterization and quantification.

Proteomics methods allow for a large number of proteins to be studied simultaneously in order to obtain accurate and comprehensive data and to correlate expression-level changes of proteins in DS pregnancies. The methods used for marker identification and validation are data mining (**chapter 10**) and bead-based multiplexed immunoassays (**chapter 11 and 12**). Furthermore, the feasibility of antibody-arrays as a high-throughput technique for DS screening was studied (**chapter 13**).

In **chapter 14** all results are summarized and discussed. Based on our conclusions, future perspectives towards prenatal screening are proposed. Ultimately, the research presented in this thesis should provide guidance towards and optimized prenatal screening programme.

Chapter 2

Down syndrome screening: imagining the screening test of the future

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Abstract

Prenatal screening for Down syndrome (DS) is performed by risk calculation based on biochemical and biometric parameters. This way, approximately 75–85% of all DS cases can be detected. A way to improve detection rates is to search for new screening markers. Since the majority of biomarkers used in current DS screening are predominantly produced by the placenta, and the presence of an extra chromosome (as in DS) complicates placental development and function, it is plausible to assume that new potential screening markers may also originate from the placenta. Any alterations in these markers can be attributed to abnormal placental development and function. This article focuses on normal early placental development and function compared with that in DS pregnancies. Using this knowledge, we reason towards candidate biomarkers that may be useful in screening for DS.

Introduction

A trisomy of chromosome 21 results in the most common chromosomal disorder in humans, Down syndrome (DS), which is present in approximately one out of 500-800 live born children [57]. Prenatal screening for DS usually consists of risk calculation based on biochemical and biometric parameters, as well as maternal age, after which women with a high predicted risk may opt for invasive testing, such as amniocentesis or chorion villus sampling. Initially, the most commonly used method for risk calculation was the second trimester triple test, which combines serum levels for alpha-fetoprotein (AFP), unconjugated estriol (uE3) and the free β subunit of human chorion gonadotropin (fβ-hCG) with maternal age [16, 32]. Currently, the most popular algorithm that is used for DS screening is the so-called first-trimester combined test, performed between 8 and 13 weeks of gestational age. This test is composed of the concentrations of pregnancy-associated plasma protein A (PAPP-A) and fβ-hCG in maternal serum, the ultrasonographic nuchal translucency measurement (NT) and maternal age [43, 58]. With the latter test, approximately 75-85% of all DS cases can be detected at a false positive rate (FPR) of 5% [53, 59, 60].

There are several ongoing studies on new non-invasive techniques for DS screening, using, for example, foetal DNA and foetal cells in maternal blood [61-63], that provide promising results for the detection of foetal DS. However, we believe that a possibly cheaper and for now more applicable method to improve first-trimester DS screening is by means of multiple marker analysis in serum. New, discriminative markers to be used for this approach can be identified using innovative proteomics approaches.

Proteomics is the large-scale study of proteins with regard to their structure, functional characterization and quantification. Proteomics methods allow for a large number of proteins to be studied simultaneously in order to obtain accurate and comprehensive data and to correlate expression-level changes of proteins. Among others, proteomics is used to detect biomarkers for a specific disease or syndrome.

Since the majority of biomarkers used in current DS screening, such as PAPP-A and fβ-hCG, are predominantly produced by the placenta it may be expected that new potential biomarkers will also originate from the placenta and that any alterations in these biomarkers might be due to abnormal placental development and function. The presence of an extra chromosome might cause deregulation and/or differential expression of different biological markers, such as proteins, cytokines and growth factors, involved in implantation and placental development, which could lead to an early disturbance of these processes [64, 65]. This might affect the placental production of other biological markers in the form of over- or under-expression of hormones and proteins. When any alterations in regulatory markers are present they might be traceable in maternal blood and could, therefore, be used as new screening markers.

In this article we will focus on normal placental development and function during early pregnancy. Furthermore, the developmental differences between chromosomally normal and abnormal placentas will be explored. Using this knowledge we will try to come up with a list of candidate biomarkers that could potentially be used in screening for DS.

Physiology of placental development

A total of 5 days after fertilization, the oocyte develops into a blastocyst. The first step of implantation takes place 6-7 days after fertilization. The blastocyst then consists of two main cell types. The trophoblast is the outer wall, surrounding the blastocystic cavity and the embryoblast is the inner cell mass. During attachment and after invasion of the endometrial epithelium, the trophoblastic cells show increased proliferation which results in a double layered trophoblast. The outer layer, facing the maternal tissue, is transformed into a syncytiotrophoblast, a continuous system not interrupted by intercellular spaces, by fusion of trophoblast cells. The inner layer, which has not yet achieved contact with the maternal tissue, is called cytotrophoblast. At approximately days 7 and 8 the mass of the syncytiotrophoblast starts to increase and achieves considerable thickness when the cytotrophoblast continues to proliferate and fuse. On day 8, small intrasyncytial cavities appear in the increasing syncytial mass forming a system of lacunae. With advancing implantation, the syncytial mass expands over the entire surface of the blastocyst. This process lasts until the blastocyst is fully implanted in the uterine wall [66]. Blindly ending syncytial branches form and protrude into the lacunae forming primary villi. In the villous phenotype, the cytotrophoblast cells of the villi (in the intervillous space) remain attached to the villous basement membrane, forming a monolayer of epithelial cells. These cytotrophoblast cells proliferate and differentiate, by fusion, to form a syncytiotrophoblast that covers the entire surface of the villus [67]. In the next step of development, the maternal perfusion is provided through the lacunar system. Shortly after the first appearance of maternal erythrocytes in the lacunae (day 13), increased cytotrophoblast proliferation takes place [68]. The cytotrophoblast cells penetrate the syncytiotrophoblast layer to form columns of extravillous trophoblast cells [69, 70]. Foetal and maternal blood come in close contact as soon as an intravillous circulation is established. However, the two bloodstreams are always separated by the placental barrier, which is composed of several layers, among which is a continuous layer of syncytiotrophoblasts and an initially discontinuous layer of cytotrophoblasts [66]. During the early stages of implantation, erosion of the maternal tissues occurs under the influence of the syncytiotrophoblast. The presence of eroding trophoblast, by being a mechanical irritant and by hormonal activity, causes the endometrial stromal cells to proliferate and enlarge to form the maternal part of the placenta which is the decidua [66]. Extravillous trophoblast cells invade the decidua and spiral arteries and play a huge role in the placental vascularization [69].

Oxygen levels also play a major role in placental development. Oxygen status is determined by the balance between reactive oxygen production and their destruction by antioxidant enzymes. The oxidative state of the cytotrophoblast is a key element in regulating differentiation into syncytiotrophoblast and indicates a role for oxygen radicals (superoxides; O_2^-) in the modulation of cell fusion [71]. Furthermore, the oxidative balance has a considerable influence on the regulation and synthesis of several growth factors [72, 73].

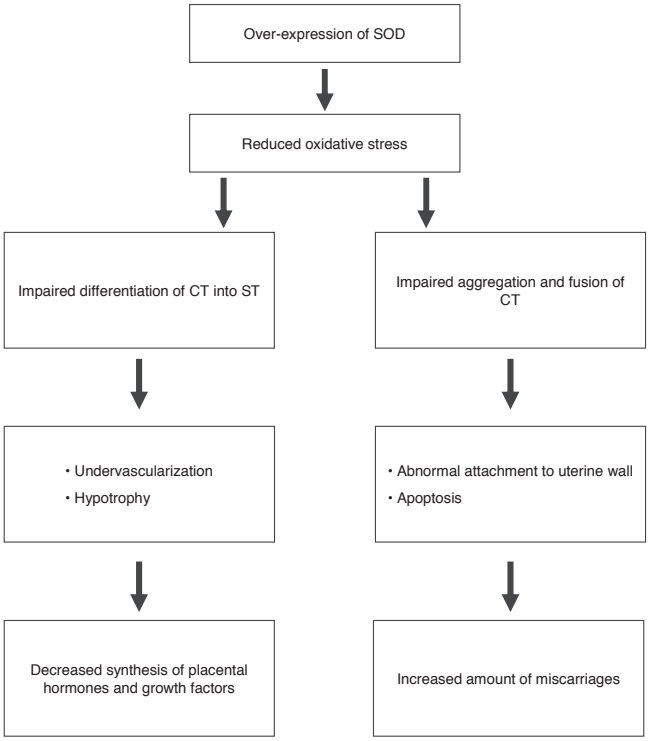

Figure 1 – Overview based on the hypothesis that over-expression of superoxide dismutase in Down syndrome pregnancies may affect placental development.
CT: Cytotrophoblast; SOD: Superoxide dismutase; ST: Syncytiotrophoblast.

Placental development in Down syndrome

Chromosomal abnormalities can cause distinct changes in placental development [74]. For trisomy 21, some characteristic pathological changes in the placenta have been described. Most of these changes are probably the result of oxidative stress, as is shown in *Figure 1*.
Oxidative stress is an imbalance between the production of intra-cellular free radicals and the capacity to detoxify them. Oxygen radicals stimulate the differentiating ability of cytotrophoblast into syncytiotrophoblast or invasive cytotrophoblast [75]. The excess of

oxygen radicals produced as reaction intermediate during oxygen metabolism must be eliminated by natural antioxidants and superoxide dismutase (SOD). The gene responsible for this reaction is Zn-SOD and is encoded by chromosome 21. SOD expression and protein levels and activity are significantly higher (about 50%) in trophoblast cells from DS placentas [76], because the extra chromosome leads to an additional gene copy. Over-expression of SOD hampers normal trophoblast formation; DS cytotrophoblast cells cannot fully compensate for the reduced oxidative stress owing to SOD over-expression resulting in an inhibited fusion and differentiation of the cytotrophoblast. The inability of cells to fuse and differentiate into syncytiotrophoblast is associated with a decrease of syncytial products, such as hormones and growth factors.

Isolated cytotrophoblast cells from normal placentas aggregate and fuse in vitro within 72 hours to form a syncytiotrophoblast. Cytotrophoblast cells isolated from DS placentas aggregate less and do not fuse well or not at all, resulting in a strongly impaired syncytiotrophoblast formation after 72 hours [77, 78]. This illustrates a decrease or delay in syncytial formation and morphological differentiation.

In DS pregnancies, it was recently found that the chorionic villi show an increased double layer of proliferative trophoblasts and an increased proportion of villus capillaries with nucleated foetal red cells [79]. In these pregnancies there is also a significant inverse association between villus diameter and number of capillaries per villus with nuchal translucency (NT) thickness [79], an established and widely used marker for DS. This suggests that abnormal placental development causing increased peripheral resistance may be an additional contributory factor to raised NT.

Even in the first trimester, the DS placenta is considerably smaller than in controls and undervascularization and hypotrophy are typical phenomena [80] However, the extent of effects on placental development vary widely.

Some characteristics of DS placentas are associated with abnormal placental attachment to the uterine wall. There is a marked fibrinoid deposition at sites of placental attachment and blood vessel invasion and a reduction in cytotrophoblasts, which often detach from the uterine wall. In cultured DS cytotrophoblasts, there are not only fewer cell aggregates, but many cells also show signs of apoptosis [81]. This could be an explanation for the high amount of miscarriages in DS (30%) [82, 83].

Notably, in other trisomies than DS, that is, trisomy 13 (T13) and trisomy 18 (T18), impaired placental development is even more pronounced. These trisomies are characterized by first-trimester intrauterine growth restriction [84], which is probably caused by poor placental development. Minguillon *et al.* studied 30 trisomic placentas from spontaneous abortions and found a defective truncal development, absent stem villi, hypovascularisation of the peripheral villi and hydropic stromal degeneration in a majority of the cases [85]. Furthermore, the placental cell proliferation rate was found to be increased in T18 pregnancies, probably as a result of increased cell death [86].

Biomarkers involved in early placental development

The formation and differentiation of the trophoblast is precisely controlled by different markers, such as hormones, growth factors and cytokines. However, the exact processes of trophoblast development are still not fully understood. The most important pathways involved in early placental development are summarized and discussed in this section and an overview is given in *Table 1*.

Table 1 – Early biomarkers involved in placental development: chromosomal origin and function according to the most important pathways.
↓: Decrease; ↑: Increase; DS: Down syndrome; T18: Trisomy 18.

Marker	Description	Chrom.	Function	Potential screening marker?
ADAM12	ADAM metallopeptidase domain 12	10	* Involved in proteolysis, adhesion, fusion and intracellular signalling * Interacts with IGF binding proteins	1st trimester: ↓ in DS and T18
EGF	Epidermal growth factor (β-urogastrone)	4	* Promotes differentiation and prevents apoptosis in trophoblasts * Involved in trophoblast invasion and proliferation	1st trimester: ↓ in DS
hCG	Chorionic gonadotropin	19	* Glycoprotein hormone that consists of a common α subunit and a unique β subunit * Stimulates the ovaries to synthesize steroids to maintain pregnancy * Involved in trophoblast differentiation and cell aggregation	Current screening marker 1st trimester: ↑ in DS, ↓ in T18
hPL	Chorionic somatomammotropin hormone 1 (placental lactogen)	17	* Member of the somatotropin/prolactin family that is expressed mainly in the placenta * Plays an important role in growth control	1st trimester: ↓ in DS
IGF-1	Insulin-like growth factor 1	12	* Regulates placental growth and transport, trophoblast invasion and placental angiogenesis * Has large effects on cell proliferation and differentiation	1st trimester: no difference
IGF-2	Insulin-like growth factor 2	11		1st trimester: no difference

IGFBP-1	Insulin-like growth factor binding protein 1	7	* Bind both IGF I and II * Restrict trophoblast invasion * Stimulate trophoblast cell migration and invasion * Regulate IGF bioavailability and cell growth	1st trimester: no difference in DS, ↑ in T18 2nd trimester: ↓ in DS
IGFBP-2	Insulin-like growth factor binding protein 2	2		1st trimester: no difference in DS, ↓ in T18
IGFBP-3	Insulin-like growth factor binding protein 3	7		1st trimester: no difference 2nd trimester: ↓ in DS
IGFBP-4	Insulin-like growth factor binding protein 4	17		Unknown
IGFBP-5	Insulin-like growth factor binding protein 5	2		Unknown
IGFBP-6	Insulin-like growth factor binding protein 6	12		Unknown
IGFBP-7	Insulin-like growth factor binding protein 7	4		Unknown
Leptin	Leptin	7	* Involved in angiogenesis, growth and immunomodulation * Regulation of foetal and uterine metabolism	1st trimester: no difference
MMP-2	Matrix metallopeptidase 2 (gelatinase A)	16	* Involved in extracellular matrix degeneration in embryonic development, reproduction and tissue remodelling	Unknown
MMP-9	Matrix metallopeptidase 9 (gelatinase B)	20	* Play a role in endometrial menstrual breakdown, regulation of vascularization and the inflammatory response * Regulate several growth factors and cytokines	Unknown

PAPP-A	Pregnancy-associated plasma protein A	9	* Cleaves IGFBPs * Important regulator of IGF bioavailability and cell growth	Current screening marker 1st trimester: ↓ in DS, ↓ in T18
PGH	Placenta-specific growth hormone	17	* Member of the somatotropin/prolactin family that is expressed mainly in the placenta * Plays an important role in growth control. * Has a key role in the control of IGF-1 levels	1st trimester: ↓ in DS
PLGF	Placental growth factor	14	* Mainly involved in angiogenesis * Has an autocrine function in regulating trophoblast function	No consensus between studies
SOD-1	Superoxide dismutase 1	21	* Binds copper and zinc ions * Responsible for catalyzing free superoxide radicals	2nd trimester: ↑ in DS
TGF-β	Transforming growth factor β 1	19	* Inhibits cytotrophoblast migration * Decreases trophoblast proliferation * Increases formation of placental giant cells * Regulates many other growth factors	Unknown
TIMP-1	Tissue inhibitor of matrix metallopeptidase 1	X	* Natural inhibitors of MMPs * Promote cell proliferation * Anti-apoptotic function * Suppress endothelial proliferation * Maintain tissue homeostasis * Regulate platelet aggregation and recruitment * Play a role in hormonal regulation and endometrial tissue remodelling	Unknown
TIMP-2	Tissue inhibitor of matrix metallopeptidase 2	17		Unknown
TIMP-3	Tissue inhibitor of matrix metallopeptidase 3	22		Unknown
TIMP-4	Tissue inhibitor of matrix metallopeptidase 4	3		Unknown
VEGF	Vascular endothelial growth factor	6	* Mediates vascular permeability * Induces angiogenesis, vasculogenesis and endothelial cell proliferation * Promotes cell migration * Inhibits apoptosis	Not detectable in maternal serum

Growth factors

Insulin-like growth factors (IGFs) are synthesized in different placental cell types, but mainly in proliferating cytotrophoblast cells [87, 88]. IGFs are involved in the regulation of placental growth, trophoblast invasion and placental angiogenesis [89], and they have a stimulating effect on cell proliferation and differentiation [90]. Specifically IGF-2 stimulates extravillous trophoblast cell migration [91].

IGFs are regulated by IGF-binding proteins (IGFBPs). There are seven different IGFBPs, of which IGFBP-1 has the greatest abundance [92]. It has been shown to restrict trophoblast invasion and is involved in cell migration [93, 94]. The second most abundant IGFBP involved in early placental development is IGFBP-4. IGFBP-4 protease, which is better known as PAPP-A, is expressed in syncytiotrophoblasts and extravillous cytotrophoblasts [95]. PAPP-A cleavage of IGFBP-4 occurs in the presence of IGF and, therefore, PAPP-A is thought to be an important regulator of IGF bioavailability and cell growth [96]. Furthermore, a disintegrin and metalloprotease-12 (ADAM12) binds to and has proteolytic activity against IGFBP-3 and, to a lesser extent, IGFBP-5. ADAMs are involved in proteolysis, adhesion, fusion and intracellular signalling [97].

Another important growth factor involved in the implantation process is epidermal growth factor (EGF). EGF is produced both in decidual and trophoblastic cells [98]. EGF promotes differentiation and prevents apoptosis induced by tumour necrosis factor alpha (TNF-α) in trophoblasts [99, 100]. Furthermore, EGF is involved in trophoblast invasion and proliferation [101, 102].

Transforming growth factor beta (TGF-β) is expressed in both endometrial and trophoblastic cells [103] and has an anti-invasive effect by inhibiting cytotrophoblast migration [94]. Furthermore, TGF-β decreases extravillous trophoblast proliferation and increases the formation of placental giant cells [104] which are aggregated trophoblast cells.

Vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) are growth factors with mainly an angiogenic effect. VEGF and PLGF are expressed in both syncytiotrophoblast and cytotrophoblast. VEGF increases the vascular permeability [105] and stimulates the vascular network in the decidual tissue by promoting endothelial cell proliferation [106]. PLGF stimulates angiogenesis. In addition, the presence of PLGF receptors on trophoblasts suggests that PLGF may also have an autocrine function in regulating trophoblast function [107].

Metalloproteases

Invasive extravillous trophoblast cells of the early placenta are embedded in a self-secreted extracellular matrix and trophoblast invasion is facilitated by degradation of this matrix [108]. Matrix metalloproteases (MMPs) are localized in the cytotrophoblast and are involved in this matrix degeneration and in the regulation of several growth factors and cytokines [103].

MMPs are a large family of enzymes that can be divided into five groups based on their specificities and location: gelatinases, collagenases, stromelysins, elastases and matrilysins. The gelatinases, MMP-2 and MMP-9, are the most common MMPs involved in placental development.

The activity of MMPs is directly inhibited by tissue inhibitors of MMPs (TIMPs). These are expressed by decidual cells and also by trophoblast cells [109, 110]. Besides the inhibition of MMPs, TIMPs have also a role in increasing cell proliferation and embryo development [111, 112].

Hormones

Hormones play an important role in the preservation of pregnancy and most are produced and regulated by the placenta. Syncytiotrophoblast formation is associated with a progressive increase of hormone production, especially of hCG. hCG is one of the most important hormones during early pregnancy. It can regulate the differentiation of cytotrophoblast, which produces little hCG, into syncytiotrophoblast, which produces a lot of hCG [113]. Its major role is the maintenance of the corpus luteum and of pregnancy. However, it is also involved in several mechanisms regulating trophoblast development, such as trophoblast differentiation and cell aggregation [68, 113]. hCG expression is regulated by a feedback loop, since binding of hCG to its trophoblast receptor down-regulates hCG synthesis [114].

Hyperglycosylated hCG (HhCG), also known as invasive trophoblast antigen, is a hCG variant that is produced only by poorly differentiated or less-invasive trophoblasts [115].

Placental growth hormone (PGH) is a hormone only slightly different from pituitary growth hormone and is produced by the syncytiotrophoblast [68]. Comparable to hCG, syncytiotrophoblast formation is associated with an increase of PGH secretion [116]. It is secreted in a non-pulsatile manner and its secretion gradually increases during pregnancy [117]. PGH is mainly involved in stimulating placental and foetal growth.

Another product of the trophoblast is leptin. The placenta expresses both leptin and the leptin receptor, which suggests that it is a target as well as a source for this hormone. Possible physiological effects of placental-derived leptin include angiogenesis, growth and immunomodulation. Leptin may also be involved in the regulation of foetal and uterine metabolism [118].

Interactions between biomarkers

The oxidative state of the cytotrophoblast is probably a key element in regulating syncytiotrophoblast differentiation [71]. A low oxygen supply up-regulates the synthesis of several growth factors, such as VEGF [119], while it down-regulates others, for example, EGF and TGF-β [120]. Low oxygen levels also stimulate the expression of hypoxia-inducible factor-1

(HIF-1), which maintains cytotrophoblast proliferation. HIF-1 expression, for its part, parallels that of TGF-β, which is an inhibitor of invading trophoblast [121, 122]. Cytotrophoblasts generate laminin, which stimulates collagenase IV activity and, thus, promotes invasion [123]. To counterbalance this activity, the cytotrophoblast also produces TGF-β, which induces the TIMPs [124].

IGFBP-1 has been shown to stimulate TIMP-1 secretion from cytotrophoblast cells [125]. IGF-2 inhibits IGFBP-1 and causes a dose-dependent inhibition of TIMP-3.

Furthermore, IGF-2 causes a downregulation of PAPP-A and, thus, a reduction of IGFBP-4 proteolysis [126]. This results in a decrease in IGF-2 bioavailability, which indicates that PAPP-A is an important regulator of IGF [96]. TIMPs inhibit the activity of MMPs and successful implantation and placentation depends mainly on the balance between these two.

The MMPs are also regulated by other pathways. Inflammatory cytokines, such as interleukins and TNF-α, have been shown to stimulate the MMP secretion and, thus, cytotrophoblast invasion, as well as IGFBP-1 and hCG. By contrast, MMP secretion is inhibited by leukaemia-inhibitory factor and TGF-β [127]. Notably, there are several MMPs and their regulation and function is MMP-specific. For example, MMP-2 and MMP-9, which are mainly involved in early placental development, induce activation of TGF-β and release IGF by degradation of IGFBPs [128, 129]. EGF acts on trophoblasts via a specific receptor (EGFR) and increases MMP-2 and MMP-9 secretion by cytotrophoblasts [130, 131]. Furthermore, EGF has been the first growth factor to show increased syncytial hormone secretion of hCG and human placental lactogen (hPL) as well as syncytialisation [100]. EGF is known to be present in high levels in the maternal circulation.

TGF-β may be an important factor in implantation and development because of its stimulation of fibronectin and VEGF [132, 133]. In vitro, TGF-β has been shown to regulate proteins, such as IGFBP-1 [134], and to indirectly inhibit MMP-9 and, thus, trophoblast invasion [135]. Moreover, TGF-β activation is facilitated by ADAM12 [136] and TGF-β inhibits the syncytial production and secretion of hCG and hPL [137]. Therefore, TGF-β could very well be an important factor controlling trophoblast invasion during early placental development. TGF-β, together with TNF-α, also stimulates VEGF expression [132]. VEGF stimulates the differentiation of cytotrophoblast into syncytiotrophoblast and its effect is inhibited by soluble fms-like tyrosine kinase-1 (sFlt-1), which acts by binding VEGF [138]. VEGF, in turn, enhances MMP-9 activity [139]. In *Figure 2*, an overview is presented of most of the biomarkers and their interactions.

The most important hormones involved in early placental development often have a direct interaction with trophoblast cells through autocrine or paracrine mechanisms. However, some hormones are known to affect other pathways. hCG stimulates the cyclic adenosine monophosphate (cAMP) pathway, which causes an increase of cAMP resulting in enhanced trophoblast invasiveness [140]. hCG has also been shown to stimulate trophoblast migration through an IGF-2 effect [141]. Furthermore, hCG increases the expression of VEGF [140] and

regulates MMPs indirectly by inhibiting MMP-activators [142].
Secretion of PGH appears to be regulated by glucose and has a key role in the control of IGF-1 levels. A low level of PGH is, thus, associated with a low level of IGF-1 [143, 144]. Leptin acts as negative predictor of PGH [102], but enhances hCG secretion and release [145, 146]. On the other hand, an inhibitory effect of hCG on trophoblastic leptin secretion has also been observed, suggesting that hCG might exert a possible negative feedback on trophoblastic release of leptin [147]. The role of hormones in placental development is also shown in *Figure 2*. Given the complex interaction of the different markers, it is, at present, not possible to indicate which markers are most promising.

Placental regulation in Down syndrome

The fact that DS is associated with (partially) defective placental development probably underlies the altered serum concentrations of current DS screening markers. It can be speculated that other markers linked to abnormal placental development might also be suitable for DS screening. The pathways described here provide knowledge on physiological placental development. Increasing insight into placental development in trisomic pregnancies could contribute to the identification of new screening markers. However, some morphological features of DS placentas are also shared by other pregnancy-associated diseases. Therefore, the potential markers described here may not be exclusively applicable for DS screening. *Table 1* summarizes the potential markers for trisomy screening.
Over-expression of SOD leads to reduced oxidative stress and, therefore, trophoblast injury. As a result, proliferation is increased and differentiation is decreased in the early placenta. Since the gene for SOD is located on chromosome 21, an elevation of SOD protein in DS pregnancies is expected. Indeed, this elevation has been shown in maternal serum, amniotic fluid and trophoblast cells from DS pregnancies [76, 148]. The elevated levels of SOD in DS may, at least in part, be responsible for the failure of cytotrophoblasts to fuse and form a defective trophoblast [71, 77]. A disturbed oxygen balance tends to up-regulate VEGF and down-regulate PLGF expression in trophoblasts [107]. The observed low expression of PLGF in DS could therefore very well be due to this imbalanced oxygen state and could hamper the maturation of trophoblastic cells [149, 150]. However, no difference was observed in placental VEGF between DS and control pregnancies.
As stated before, cytotrophoblast cells of DS placentas are liable to apoptosis. Since EGF prevents apoptosis, EGF levels in DS are expected to be low and the potential of EGF as a screening marker is conceivable. One study by our group indeed showed decreased EGF levels in maternal serum from DS pregnancies [151]. IGF and IGFBPs are mainly involved in foetal growth and are also mediators of placental development. Significant differences for IGFBP-1 and IGFBP-3 between DS and normal pregnancies have been described in the second

trimester [152]. However, a study by Miell *et al.* did not show any significant differences in DS pregnancies, but did find significantly increased IGFBP-1 and significantly decreased IGFBP-2 levels in T18 pregnancies [153]. Considering that growth restriction and impaired placental development are more pronounced in T18 compared with DS, these findings seem rational. The proteolysis of IGFBP stimulates growth by increasing levels of bioavailable IGF-1 and IGF-2. The IGFBP protease PAPP-A is one of the current DS screening markers in the first trimester of pregnancy and is highly decreased in DS pregnancies. The PAPP-A complex is synthesized by the placenta and secreted primarily into the maternal circulation. In normal pregnancy, PAPP-A levels in maternal blood are first measurable at approximately 8 gestational weeks and increase throughout gestation, more-or-less proportional to the size of the placenta. Similarly, ADAM12 is an IGFBP protease synthesized by the placenta and is, therefore, an obvious candidate for investigation as a predictor of chromosomal abnormalities. ADAM12 is one of the proteins that have recently been explored as potential screening markers and significant differences between DS and control pregnancies have been found [154, 155].

DS is associated with a reduced synthesis and secretion of placental hormones, such as hCG, hPL and leptin [76]. An impaired trophoblast development and function leads to a decreased syncytial production of hCG (one of the widely used current DS screening markers). Moreover, this decreased production is for its part related to abnormal formation of syncytiotrophoblast [77, 78]. Trophoblast cells from DS pregnancies produce weakly bioactive and abnormally glycosylated hCG [156]. However, maternal serum hCG levels are elevated in pregnancies associated with DS. The most plausible explanation for this contradictive phenomenon is that abnormal hCG leads to an impaired placental uptake of hCG by its receptors resulting in high maternal serum levels. Another explanation might be that higher concentrations of hCG could be due to immaturity of cytotrophoblast cells, which are unable to form a proper syncytiotrophoblast and to downregulate their own hormone synthesis. DS pregnancies are marked by poor trophoblast differentiation, which results in the accumulation of cytotrophoblast cells that produce HhCG. Significantly elevated levels of HhCG have been found in DS pregnancies compared with controls [157, 158]. However, HhCG will probably not be an additional marker in the current screening test because of the high correlation with other forms of hCG that are already part of the DS screening (total hCG and fβ-hCG). In placentas from DS pregnancies, the differentiation of cytotrophoblast into syncytiotrophoblast is impaired and the in vitro synthesis of hPL and PGH is reduced markedly [77]. If these in vitro findings are representative of in vivo trophoblast differentiation into syncytiotrophoblast, it is likely that the maternal serum concentrations of hPL and PGH will be lower in DS pregnancies and that both may be potential maternal serum markers of foetal DS. Lower values have indeed been found by Christiansen *et al.* [159, 160]. A similar reasoning would apply to leptin; however, a significant decrease in maternal serum concentrations of this hormone has not been found [161].

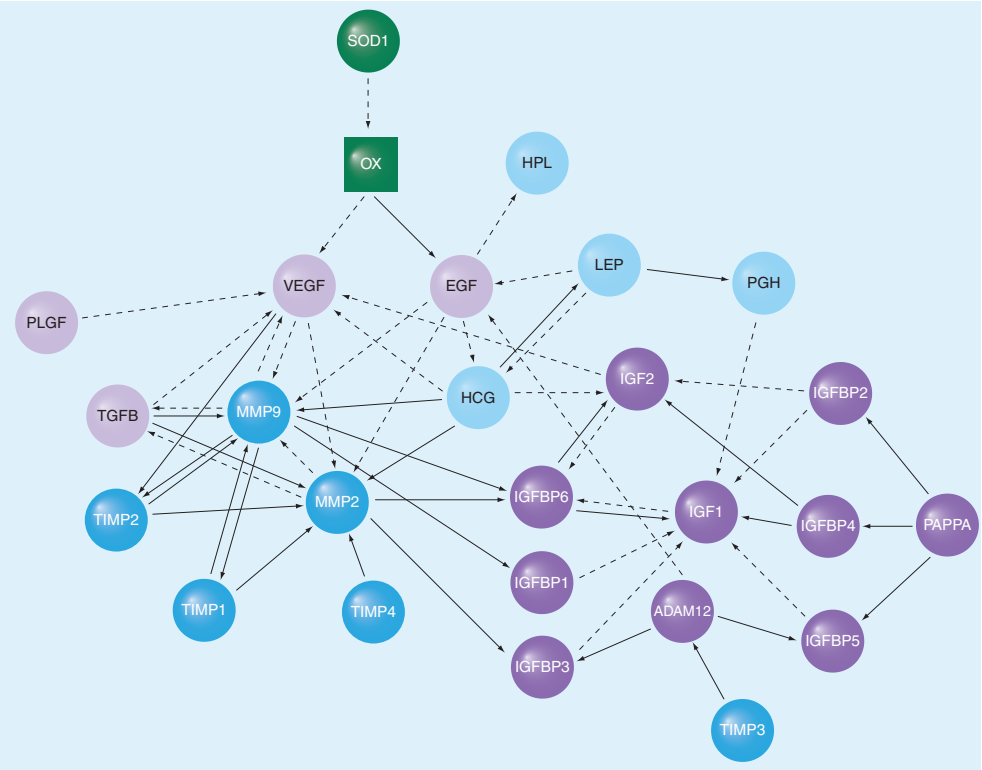

Figure 2 – Overview (created with Cytoscape) of the most important pathways involved in placental development. Pathways were explored using literature and by analyzing all markers with pathway analysis software (MetaCore, GeneGo Bioinformatics, MO, USA). Light purple: Growth factors; Dark purple: IGF pathway; Dark blue: Metalloproteases; Light blue: Hormones; Dashed arrows: Upregulation; Solid arrows: Downregulation.
OX: Oxidative imbalance.

Chromosomal disorders are correlated with abnormal maternal blood levels of growth factors, which may impair trophoblast function. As stated before, DS is associated with various defects in trophoblast differentiation and increased apoptosis. This is represented by an alteration of several agents, including upregulation of MMP-9. It is possible that cytotrophoblasts from DS pregnancies upregulate MMPs to compensate for their lower expression of adhesion molecules, which also play a critical role in invasion [81]. Recently, MMP-9 has been proposed as a potential marker for pre-eclampsia screening [162]. However, to our knowledge, no study has yet been performed to explore MMPs as potential screening markers for trisomies.

Biomarkers and Down syndrome screening

Placental development is complex and many physiological pathways are involved. The presence of an extra chromosome, as in DS, affects placental development and function. In this article, the main pathways and regulators of early placental development have been explored and the influence of DS has been evaluated. Using this knowledge we have reasoned towards candidate biomarkers that may be useful in the prenatal screening for DS. Some biomarkers we have described are already widely used as screening markers, while others have been explored as potential screening markers. However, some of these biomarkers have only been described in one or two studies. The pathways discussed here are, in our view, the most important in placental development. Nevertheless, more placental products and regulators are involved, for example, cytokines and transcript factors [163, 164]. However, we feel that these products are too complex or non-specific to be suitable as potential screening markers and were, therefore, beyond the scope of this article.

The search for biomarkers to detect DS is not an easy task. A first requisite of a suitable potential screening marker is that it is detectable in maternal blood. Second, a screening marker has to be able to distinguish DS from non-DS with high specificity. The secretion of a protein or hormone is possibly not restricted to the placenta, potentially hiding the discriminative character of such a biomarker. IGF, for example, is involved in several other pathways in the human body. Consequently, an alteration of IGF in the blood of a pregnant woman would not necessarily be due to placental dysfunction. Many potential biomarkers described will probably turn out to be non-specific for DS screening, owing to maternal expression or over- or under-expression in other pregnancy-associated diseases. Nevertheless, based on the hypotheses presented here, these biomarkers do deserve further consideration as potential DS screening markers.

Prenatal screening should preferably take place in the first trimester of pregnancy. Since the placenta is still small at early gestation, concentrations of biomarkers are relatively low and alterations may be difficult to quantify in maternal blood. Some biomarkers may, therefore, be more suitable as screening markers later in pregnancy. On the other hand, there are also screening markers that are significantly decreased in the first trimester but tend to normalize towards the second trimester. In DS pregnancies this has been described for PAPP-A [165]. Furthermore, concentrations of Placental Protein 13 (PP13) have been found to be decreased in the first trimester, while increased in the third trimester in pre-eclamptic pregnancies in which placental development is frequently also impaired [166]. Therefore, the timing of performing the actual screening test determines the biomarkers to be used.

Except for SOD, none of the biomarkers described originate from chromosome 21. Therefore we hypothesize that the impaired placental development in DS may be due to an oxidative imbalance caused by over-expression of SOD. However, impaired placental development is

even more pronounced in T18 and T13 pregnancies and none of the biomarkers originate from chromosome 18 or 13 either. It is likely that there are more complex effects in trisomic pregnancies causing an impaired foetal and placental development.

Strikingly, there are biomarkers that are highly comparable in origin and function, but behave differently in DS pregnancies. ADAM12 for example, is very similar to PAPP-A but is decreased to a much lesser extent. However, despite their similarities in origin and function, both biomarkers are regulated in different ways (*Figure 1*).

Part I | Chapter 3

Lack of standardization in determining gestational age for prenatal screening

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Abstract

Objectives To determine whether estimation of gestational age (GA) in the context of first-trimester Down syndrome screening is standardized in the Netherlands.

Methods This was a retrospective study, carried out between January 2005 and December 2006, of women who underwent first-trimester Down syndrome screening (n = 40,730) based on maternal age, maternal serum analysis and nuchal translucency (NT) measurement. Date of the last menstrual period (LMP), dating scan information including measurement of crown–rump length (CRL), NT thickness and name of the sonographer were recorded for all pregnancies. The accuracy of estimation of GA was evaluated by comparing the GA based on the LMP with that estimated from the CRL, using relevant subsets of the database. A survey of 104 sonographers was performed to further investigate the findings of the preceding analysis.

Results In 44% of all first-trimester combined tests the estimation of GA was based on the dating scan; the method of determination of GA was unknown in 23%. In 15% of all cases a dating scan was recorded but was not used to provide the estimation of GA at blood sampling. Detailed analysis showed that a consistent methodology for the estimation of GA from CRL was not maintained within hospitals and obstetric practices. For a single CRL, the reported GA differed by up to 10 days. Finally, it was demonstrated that individual sonographers reported different GAs for a given CRL.

Conclusions Currently, estimation of GA in the first-trimester in the Netherlands is not standardized. To improve the performance of prenatal screening for Down syndrome, estimation of GA should be based on ultrasound examination, with one nationally accepted CRL curve.

Introduction

First-trimester ultrasound examination is common practice for confirming the intrauterine location of a pregnancy and determining its gestational age (GA), and for measuring nuchal translucency (NT) as part of the first-trimester assessment of risk for chromosomal abnormalities.

Since 1 January 2007 all Dutch women have been eligible to apply for a first-trimester non-invasive screening test consisting of the evaluation of maternal age, concentrations of pregnancy-associated plasma protein A (PAPP-A) and free beta human chorionic gonadotropin (fβ-hCG) in maternal serum, and NT measurement. Values of the latter three parameters do not remain constant throughout pregnancy and risk estimation for Down syndrome uses standardized values based on GA. The precise determination of GA is therefore essential.

Estimation of the GA based on ultrasound measurement of the crown–rump length (CRL), if measured correctly, has been shown to produce a more reliable estimate of the GA than calculation based on the first day of the last menstrual period (LMP) [167, 168]. The CRL reference curve described by Robinson and Fleming in 1975 is generally recommended [169]. However, this curve is not unambiguous, because Robinson and Fleming corrected their original curve for systematic measurement errors [169]. The difference between the corrected and uncorrected curves is 1–2 days of GA for a given CRL. In practice, the existence of two Robinson and Fleming reference curves is not generally known. Moreover, since 1975 many other reference curves for GA based on CRL have been produced and a meta-analysis of 21 different curves has been published recently [170].

The fact that there are two methods for estimating GA (LMP and by measuring CRL), and that there are a number of different reference curves for estimating GA from CRL, raises questions about the extent to which the determination of GA is standardized. We performed a retrospective analysis of the estimation of GA from CRL in the Netherlands in 2005–2006, using data obtained from requests for first-trimester combined tests for Down syndrome. In addition, a survey was carried out among healthcare professionals on the subject of the reference curves used to estimate GA from CRL.

Methods

Between January 2005 and December 2006, the National Institute for Public Health and the Environment (RIVM) in Bilthoven, the Netherlands, received maternal blood samples and foetal NT measurements from 72 centres for prenatal screening. Only singleton pregnancies for which the CRL (at the time of the NT measurement) was between 38 and 84 mm were included in this study. Moreover, applications from centres sending fewer than 10 samples were excluded.

Samples were accompanied by a form that recorded relevant information concerning the pregnancy (*Figure 1*). The most important detail on this form was GA on the day of blood sampling (GA-blood sampling). The health professionals who completed the forms were free to choose the method of calculation for the GA-blood sampling, either LMP or ultrasound dating. Additionally, those completing the forms were asked to give the date of the LMP (with which to calculate the GA based on the LMP; GA-LMP) and the GA based on a dating scan (GA-dating scan). The NT and CRL, mostly accompanied by the date of the NT measurement and the name of the sonographer, were also included in the appropriate box on the form (*Figure 1*). In this study, the CRL was converted into GA (GA-CRL) using the uncorrected formula of Robinson and Fleming (GA-CRL = 8.052√CRL + 23.73).

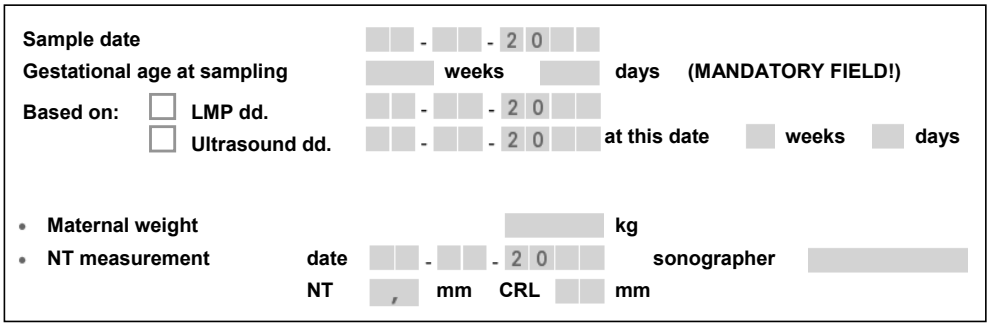

Figure 1 – Pregnancy information form, including gestational age, crown–rump length (CRL), date of last menstrual period (LMP) and nuchal translucency thickness (NT).

To study the level of standardization the percentages of GA-blood sampling based on GA-LMP and on GA-dating scan were calculated. To investigate the correct use of reference curves, the CRL was compared to the GA-dating scan. Because the available data only included a CRL value obtained at the time of the NT measurement there was no direct relationship between the CRL and the GA-dating scan (*Figure 1*). Therefore, as an alternative approach, only forms in which the dating scan and the NT measurement were performed on the same day were included. To clarify how the GA-dating scan was used, the CRL, measured at the time of the NT measurement, was compared to the GA-dating scan. For this analysis, data were obtained from four large centres for prenatal screening (>400 NT measurements per year). The relationship between CRL and GA-dating scan for each of the four centres was determined by means of polynomial regression analysis. The resulting curves were compared with the reference curves of Robinson and Fleming. It was presumed that within one centre the same reference curve was used by all sonographers.

Next, the difference between the GA-CRL and GA-dating scan was calculated for the entire study population and compared with the reference curves of Robinson and Fleming. Only

deviations between ± 5 days were included in this analysis. The mean deviation and SD were also based on this selection only. Larger differences occurred very infrequently. Because it was difficult to establish whether the larger differences were actual or the result of measurement/writing errors, they were excluded from this analysis.
To elucidate the differences between the GA-dating scan and the GA-CRL, an e-mail survey was performed among 104 operators from 38 centres. An inventory of the type of ultrasound equipment was made and operators were asked to calculate GA based on three CRL values (45, 60 and 75 mm). Finally, operators were asked to specify the CRL reference curve that they used to calculate GA.

Results

Between January 2005 and December 2006, 40,730 serum samples from a first-trimester screening test were included in the study population. In 44% of these cases GA-blood sampling corresponded to GA-dating scan and 33% corresponded to GA-LMP. In the remaining 23% of cases, it was unclear whether GA-blood sampling was based on the dating scan or LMP, partly because the GA-LMP and the GA-dating scan were both unknown and partly because the GA-LMP and the GA-dating scan were the same. Thus, a dating scan was used to determine the GA-blood sampling at most in 67% of cases (*Table 1*).

Table 1 – Overview of the use of the dating scan or last menstrual period (LMP) to determine gestational age (GA).

Parameter	*n* (%)
Total study population	40730*
Dating scan filled in on application form	23966 (59)
LMP filled in on application form	20790 (51)
Dating scan used to determine GA	17906 (44)
LMP used to determine GA	13515 (33)
Unknown source used to determine GA	9309 (23)
GA-dating scan and GA-LMP the same	4167 (10)
GA-dating scan and GA-LMP unknown or incorrect †	5142 (13)

**Singleton pregnancies in which GA was between 56 and 100 days.*
†Not filled in on the application form or different from the GA based on blood sampling.
GA-dating scan, GA based on dating scan; GA-LMP, GA based on LMP.

The GA-dating scan, as given on the application form, was not always used to derive the GA-blood sampling. In 15% of cases a substantial difference between the two (up to 10 days) was present. There was no obvious relationship between the difference in days and choice of using either LMP or dating scan to determine GA.

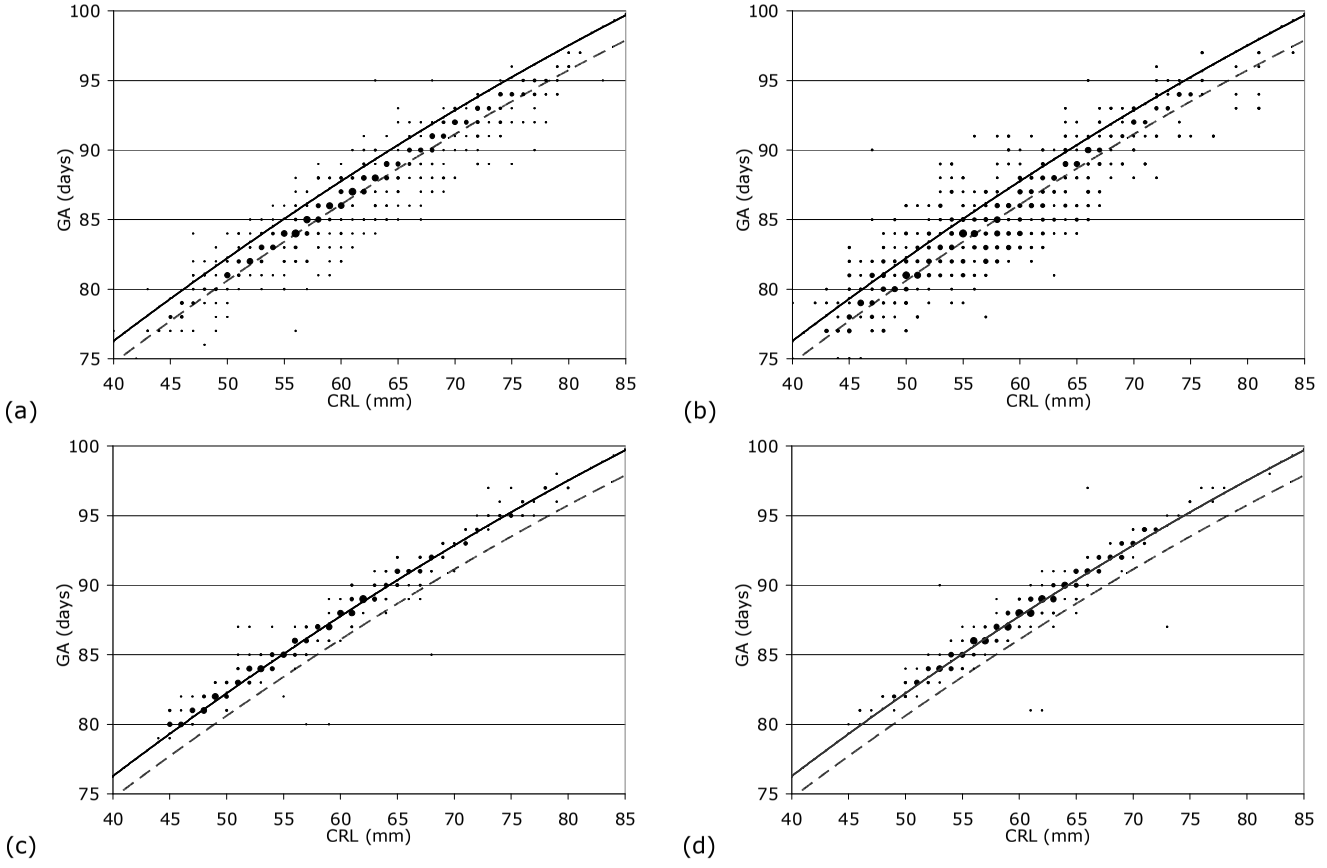

Figure 2 – Gestational age (GA) based on dating scan plotted against crown–rump length (CRL) measurements obtained on the same day in four large centres for prenatal screening in the Netherlands. Values are compared with both the uncorrected (dashed line) and corrected (solid line) reference curves of Robinson and Fleming. Total number of measurements was 791 in Centre A (a), 675 in Centre B (b), 469 in Centre C (c) and 444 in Centre D (d). Size of the dot represents the relative number of measurements at each point.

The resulting curves for the four large centres for prenatal screening showed clear differences between the centres (*Figure 2*). For Centres C and D the variation was rather small (up to 5 days for a given CRL), whereas for Centres A and B it was much larger (up to 10 days for a given CRL). For Centres A and B the statistically modelled relationship between CRL and the GA-dating scan matched the uncorrected Robinson and Fleming reference curve, whereas for Centres C and D it matched the corrected Robinson and Fleming curve.
Table 2 shows the difference in days between GA-dating scan and GA-CRL calculated with the uncorrected as well as the corrected Robinson and Fleming formulae. Mostly, a difference of 1 or 2 days was seen with both.

Table 2 – Difference in days between gestational age (GA) based on dating scan and that based on crown–rump length (CRL) calculated with the uncorrected and corrected Robinson and Fleming curve.

	Robinson and Fleming curve (*n* (%))	
Difference (days)	Uncorrected (*n* = 8857)	Corrected (*n* = 8833)
-5	96 (1.1)	23 (0.3)
-4	196 (2.2)	57 (0.6)
-3	654 (7.4)	130 (1.5)
-2	2867 (32.4)	315 (3.6)
-1	2203 (24.9)	1228 (13.9)
0	1583 (17.9)	3268 (37.0)
1	569 (6.4)	1848 (20.9)
2	325 (3.7)	1058 (12.0)
3	243 (2.7)	435 (4.9)
4	84 (0.9)	315 (3.6)
5	37 (0.4)	156 (1.8)
Mean (SD) days	-1 (2.1)	1 (2.1)

CRL measurement and GA dating scan were performed on the same date. Differences over ± 5 days were excluded from analysis.

Of all the operators who participated in the survey, a complete response was received from 24, representing 21 centres. A total of 14 different ultrasound devices were used. Over 90% of the respondents indicated that they used Robinson and Fleming's reference curve. For this group the mean GA and range for a given CRL were calculated. The results were compared with the corresponding GA derived by applying the Robinson and Fleming curves (*Table 3*). In addition, two operators, one from a centre where Robinson and Fleming curves are also used, indicated that they used the reference curve of Hadlock *et al.* [171]. Both operators reported a GA of 79 days, 87 days and 95 days at CRL values of 45 mm, 60 mm and 75 mm respectively.

Discussion

In this study we found that, in the Netherlands, standardization of the estimation of GA is lacking. In only 44–67% of cases was GA based on a dating scan, and in 15% of cases in which a dating scan was performed it was apparently not used to determine the GA-blood sampling. In large centres for prenatal screening the GA-blood sampling and the GA-dating scan differed by up to 10 days. The causes of this may include the use of LMP for dating, the use of different CRL reference curves and calculation or clerical errors.

In two of the four centres for prenatal screening the relationship between GA-dating scan and the GA-CRL appeared quite standardized but in the other two centres the variation in GA for a given CRL was considerable. Moreover, in two centres, the data fitted the uncorrected Robinson and Fleming curve, whereas in the other two they fitted the corrected Robinson and Fleming curve. There are a number of explanations for these discrepancies. In this study it was assumed that the GA-dating scan and the GA-CRL, when performed on the same day, were in fact the same ultrasound examinations. Although likely, this is not necessarily true. Of course, two separate CRL measurements, one performed for a dating scan and the other for an NT measurement, may result in some inter-sonographer variation. Additionally, the reference curves for the two sonographic devices may differ. Finally, differences of 1 day may have been produced by arithmetic rounding.

Table 3 – Determination of gestational age in the Netherlands by centres that use a Robinson and Fleming reference curve.

		Gestational age (days)	
		Robinson and Fleming curve	
CRL (mm)	Mean (range)	Uncorrected	Corrected
45	79 (78-81)	78	79
60	87 (84-89)	86	88
75	95 (93-96)	93	95

CRL, crown–rump length.

Nevertheless, even when the dating scan and the NT measurements were two separate events, the differences should not have been as striking as those seen in *Figures 2a-b*. Regression analysis of the data in *Figure 2* indicates that, in practice, both Robinson and Fleming curves are applied, because the data from two centres fit the corrected curve and the data from the other two fit the original curve.

The data already showed an inconsistent relationship between GA-dating scan and GA-CRL based on the reference curve of Robinson and Fleming. The e-mail survey corroborated this finding. All but two of the operators used a Robinson and Fleming curve to calculate GA

from a given CRL (but did not specify whether the original or corrected reference curve was used). The range of GA for a given CRL value was up to 3 days and this may be due to the fact that the two different Robinson and Fleming reference curves were applied.

Based on these results we conclude that standardization of GA determination at blood sampling for first-trimester screening tests is insufficient. A valid dating scan is not used frequently enough and, when a dating scan is performed, the underlying reference curves used to estimate GA from CRL differ. In the risk estimation for Down syndrome of an individual pregnancy, an incorrect GA may lead to an erroneous high risk or non-high risk outcome of the test [172, 173]. It might be better to relate PAPP-A and fβ-hCG serum concentrations to CRL values, instead of GA. Thus, miscalculations owing to inaccuracies or lack of standardization in the translation of CRL into GA would be avoided. Whether this will actually improve the performance of Down syndrome screening is the subject of an ongoing study at our laboratory.

In a very small percentage of pregnancies intrauterine growth restriction may be present in the first trimester, for example in cases of trisomy 13 and 18. This could lead to an underestimation of GA if dating scan methodology is used. A comparison of the GA-LMP and GA-dating scan can obviate this. In general, however, GA should be determined by dating scan using a single CRL reference curve.

Acknowledgments

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Part I | Chapter 4

Quality of nuchal translucency measurements in the Netherlands; a quantitative analysis

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Abstract

Objective The Dutch Centre for Population Research has specified quality demands for nuchal translucency (NT) measurements in The Netherlands. We performed an analysis of the quality of NT measurements in 2005–2006 and its influence on screening performance.
Methods This was a retrospective study of records of NT measurements (n = 27,738) obtained between January 2005 and December 2006 retrieved from the Dutch National Institute for Public Health and the Environment (RIVM). The performance of each individual operator was analyzed with regard to the quality standards, which involved calculation of operator-specific median NT multiples of the median (MoM) values. For the entire population of operators, a curve was determined describing the relationship between crown–rump length and NT. Detection rates (DR) and false-positive rates (FPR) for Down syndrome were modelled with this new curve and compared to those originally obtained using previously published reference data.
Results Only 22% of all operators met the quality requirement of performing more than 150 NT measurements per year. However, no relationship was found between the number of measurements per operator and their median NT-MoM. The mean of all operator-specific median NT-MoM values was 0.94 (target value 1.0). Overall, operators with a Fetal Medicine Foundation certificate measured a significantly higher median NT-MoM (mean of operator-specific medians, 0.98) as compared to the non-certified operators (0.92). During the study period, the monthly median NT-MoM of all operators rose steadily, from 0.86 in January 2005 to 0.96 in December 2006. Recalculation of the risk for Down syndrome after adjusting the reference NT medians using our own data led to a modelled 4% increase in DR at a 5% FPR.
Conclusion Improved monitoring of NT measurement put into effect during the study period seems to have led to an improvement in the accuracy of measurements. Strict quality demands, continued monitoring and scrupulous evaluation of individual operators is likely to lead to an even better performance.

Introduction

The first-trimester combined screening test is composed of the measurements of pregnancy-associated plasma protein A and the free beta subunit of human chorionic gonadotropin levels in maternal serum combined with a nuchal translucency (NT) measurement and maternal age [58, 174]. Since 1999 the first-trimester combined test has been performed on an increasingly large scale in the Netherlands.

The importance of quality assurance in Down syndrome screening has been mentioned in several reports of the Dutch Health Council [50, 51]. The Health Council stated that good quality screening, especially for the quality of NT measurements, is only possible within an organized programme. In accordance with this advice a policy for nationwide screening was set up in which the Centre for Population Research was appointed as the coordinating agent [52]. The Centre for Population Research quality demands include that an operator must perform at least 150 NT measurements each year and that they need to be qualified for measuring NT through certification provided by the Fetal Medicine Foundation (FMF) or other accredited educational organizations [175]. This programme was set up in 2006 and fully implemented in January 2007.

Two types of quality assurance can be distinguished, both of which have been applied to the measurement of NT [53, 176, 177]. With qualitative assurance, aspects which contribute to adequate NT measurement, for example a correct sectional plane view of the foetus or calliper placement, are evaluated through the submission of ultrasound prints by the operator to an examining body; based on scores given by experts the quality of NT measurements can be determined [178]. The second aspect of quality assurance is quantitative. Individual operator-specific crown–rump length (CRL)/NT curves can be produced and compared to an internationally accepted reference curve [179, 180]. Additionally, calculation of multiples of the median (MoM) values provides a useful parameter that can be used to assess variation between operators. The median NT-MoM value should be 1.0 if the NT measurements of a single operator or group of operators do not deviate from the modelled median reference curve [181-184]. To ensure good quality NT measurements, the accepted median NT-MoM of an operator should be limited, e.g. to between 0.9 and 1.1, and the accepted 5^{th}–95^{th} percentile range should also be limited.

The NT measurement is a dominant parameter in prenatal screening for Down syndrome. Unsatisfactory quality of NT measurements can easily lead to over- or underestimation of the risk for Down syndrome; this underlines the importance of good quality assurance.

In this study, a quantitative analysis is presented of the quality of NT measurements over a 2-year period. Furthermore, NT-MoM values were recalculated using a new CRL/NT curve fitting this population. The screening performance that would have been obtained using these NT-MoM values was calculated, and the modelled change in the screening

performance was used to estimate the effect that can be expected from standardizing NT measurements so that the observed NT-MoM distribution matches the reference formula used.

Methods

This was a retrospective data analysis performed to assess the quality of NT measurements carried out between January 2005 and December 2006 in the Netherlands. Records were retrieved from The Dutch National Institute for Public Health and the Environment (RIVM), which received the details of 27,738 foetal NT measurements from singleton pregnancies with a CRL between 45 and 84 mm in the specified time period. The names of the operators were known for 10,845 of these NT measurements. Only operators with more than 25 NT measurements recorded over a period of at least 3 months were included in the analysis. The number of measurements was transformed to measurements per year.

Median NT-MoM values and 5th and 95th percentiles were calculated for each operator. As a reference for the expected median NT, the formula of Nicolaides *et al.* was used: $\log_{10}(NT) = -0.3599 + (0.0127 \times CRL) - (0.000058 \times CRL^2)$ [179]. Differences in medians were then compared between operators in relation to the number of measurements performed per year (stratified into 25–75, 76–150 and >150 measurements per year) using ANOVA (statistical package 'R', The R Foundation, Vienna, Austria).

To obtain an indication of the performance of all operators together, the median NT-MoM values, calculated using the curve of Nicolaides *et al.*, of all operators were averaged. A new CRL/NT curve was recently published by Spencer *et al.* [180]. To compare the two curves, NT-MoM values were also calculated using a statistical approximation of the latter curve ($NT = -(0.000456 \times CRL^2) + (0.077012 \times CRL) - 1.455088$) [185]. Furthermore, all NT-MoM values were log transformed and monthly mean $\log_{10}$(NT-MoM) values (using the Nicolaides *et al.* curve) and confidence intervals (mean ± 1.96 SEM) were calculated to evaluate their change over time throughout the study period.

To investigate the possible relationship between FMF-certification and quality of NT measurement, an inventory was made of all FMF-certified operators using the registration list of the Dutch FMF [186]. The difference between the NT measurements of operators with and without FMF-certification was statistically tested. For the six operators who performed the most NT measurements individual CRL/NT curves were produced and these were compared to the reference curves of both Nicolaides *et al.* and Spencer *et al.* Finally, a CRL/NT curve for the entire population of operators was derived, using second-order polynomial regression analysis (Microsoft Office Excel, Microsoft, Redmond, WA, USA). Based on this new curve NT-MoM values were recalculated for all the pregnancies in the study. CRL measurements were stratified into 5-mm intervals, and for each CRL interval the median and 5th, 95th and

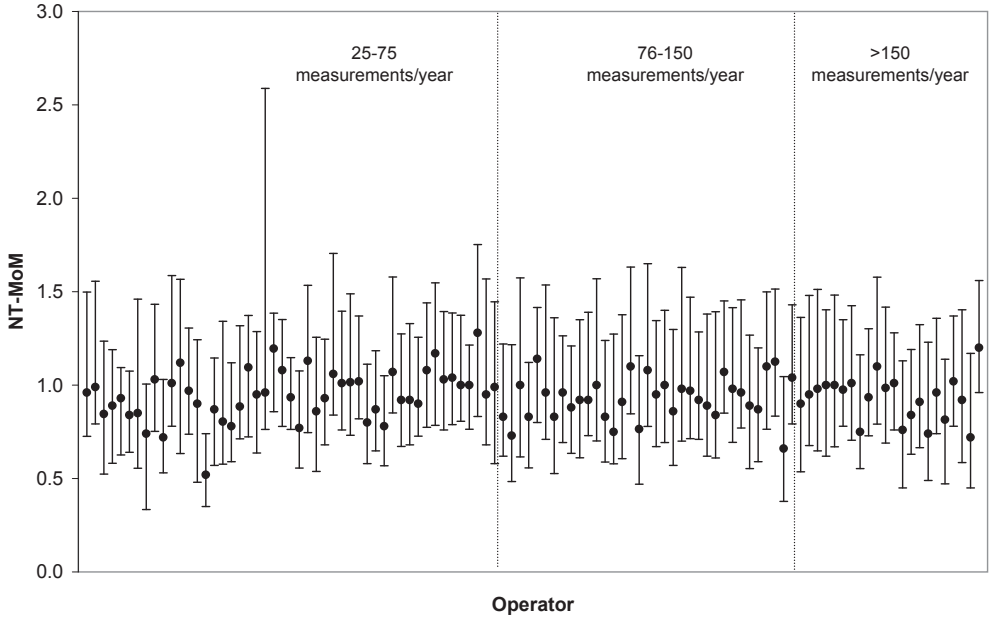

Figure 1 – Median nuchal translucency multiples of the median (MoM) values and 5th –95th percentiles for each operator, sorted by number of nuchal translucency measurements per year.

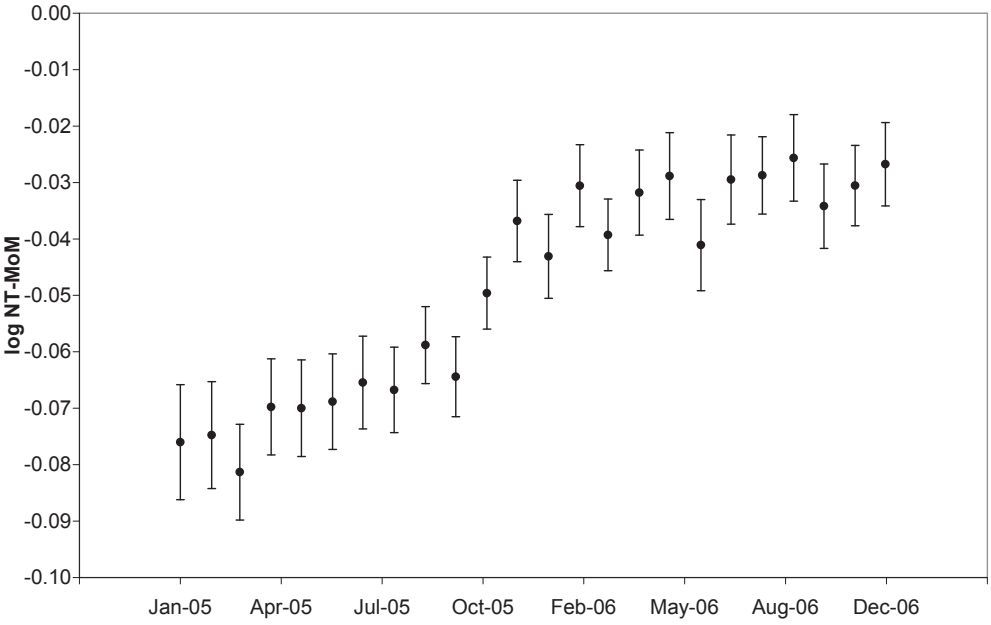

Figure 2 – Mean log-transformed nuchal translucency (NT) multiples of the median (MoM) values and 95% confidence intervals (mean ± 1.96 SEM) of all nuchal translucency measurements for each month in the study period.

99th percentiles of NT were calculated, expressed both as absolute values and MoMs using the new reference curve. A new risk for Down syndrome was estimated for each foetus using the new curve, and detection rates (DR) and false positive rates (FPR) were modelled (DSQA Tools, Media Innovations Ltd., Leeds, UK) for a comparison with the screening performance obtained using the Nicolaides *et al.* CRL/NT curve.

Results

We retrieved the records of 106 operators who had performed more than 25 NT measurements over a period of at least 3 months during the study period. Only 23 of these (21.7%) performed more than 150 measurements per year. To determine whether the number of NT measurements performed influenced the quality of the measurements, median NT-MoM values and 5th–95th percentiles of individual operators were compared to their number of measurements (*Figure 1*). From this figure it can be seen that the number of measurements per year performed by each operator was not related to their proximity to the target value of median NT-MoM (i.e. 1.0). The median NT-MoM was higher than 1.1 for nine operators (8.5%), while it was lower than 0.9 for 36 operators (34.0%). Moreover, there was no statistically significant relationship between the number of measurements per operator and medians or 5th and 95th percentiles.

Table 1 – Median and 5th, 95th and 99th percentiles of nuchal translucency (NT), expressed as absolute values and multiples of the median (MoM), calculated for measurements obtained according to 5-mm intervals of corresponding crown–rump length (CRL).

		Median		5th percentile		95th percentile		99th percentile	
CRL (mm)	*n*	NT (mm)	NT (MoM)	NT (mm)	NT (MoM)	NT (mm)	NT (MoM)	NT (mm)	NT (MoM)
45-49	2342	1.2	0.97	0.7	0.59	2.0	1.63	3.4	2.73
50-54	4297	1.2	0.95	0.7	0.55	2.0	1.51	2.9	2.15
55-59	5899	1.4	0.97	0.8	0.57	2.1	1.53	2.8	2.04
60-64	6080	1.5	1.00	0.9	0.60	2.3	1.52	2.9	1.93
65-69	4543	1.6	1.01	0.9	0.59	2.3	1.50	2.9	1.84
70-74	2794	1.6	1.00	1.0	0.61	2.5	1.53	2.9	1.82
75-80	1445	1.6	0.98	1.0	0.61	2.4	1.47	3.1	1.85
80-84	338	1.6	0.96	1.0	0.60	2.4	1.44	2.9	1.71

MoM values were calculated using the CRL/NT curve derived from our own data.

Based on the individual NT values, calculations were made regarding the entire population of operators. The mean operator-specific median NT-MoM value was 0.94. When medians were recalculated using the reference formula of Spencer *et al.* the same analysis resulted in

a mean median of 0.98 NT-MoM. The change in the mean $\log_{10}$(NT-MoM) over time is shown in *Figure 2*. There was an evident trend in monthly mean NT-MoM values during the study period, from 0.86 in January 2005 to 0.96 in December 2006.

Next, the relationship between FMF certification and NT measurement was evaluated. Of all 106 operators, 42 (39.6%) were known to be FMF-accredited. The mean median NT-MoM for this group was 0.98, compared to 0.92 for operators without FMF certification (P = 0.003, t-test). *Figure 3* shows the CRL/NT curves of the six operators, with or without FMF certification, who performed the most NT measurements per year. The plotted CRL/NT curves of certified operators were all above the previously published reference curves used in this study, while the curves of the non-certified operators were all below. Nevertheless some curves, in both groups, showed considerable deviation from the reference curve. These large deviations raise questions about the accuracy of these reference curves for the Dutch population, although the differences seem to be at least partly due to a lack of adequate standardization and quality control of NT measurements in the studied period (particularly in the case of measurements obtained early on in the period). Therefore, a new CRL/NT curve was derived using all NT measurements included in this study taken together. This curve, the mathematical formula for which is: $NT = -(0.0003 \times CRL^2) + 0.0525 \times CRL - 0.6133$, is shown in *Figure 4*.

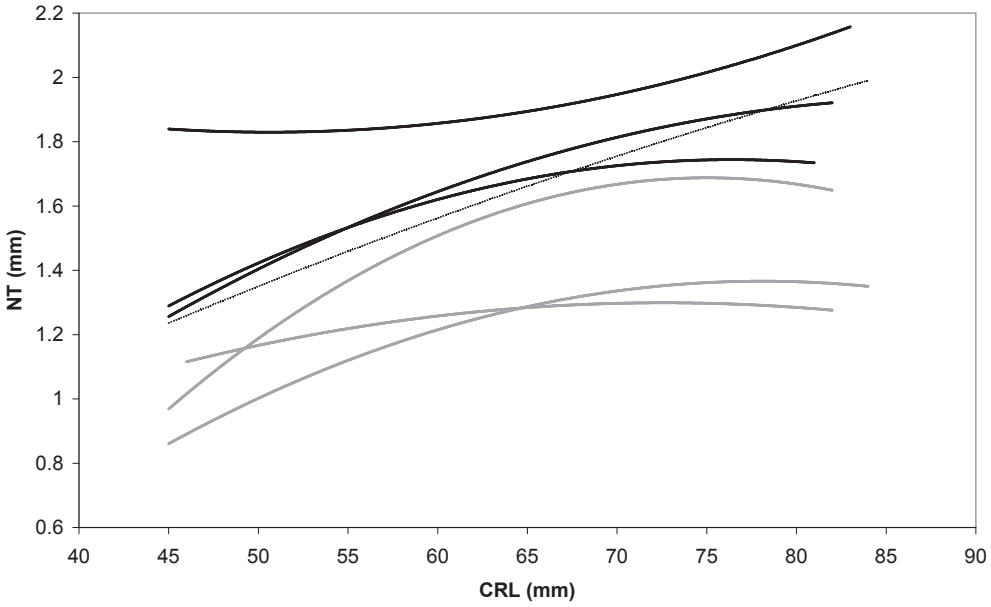

Figure 3 – Crown–rump length/nuchal translucency curves of the six operators with the most nuchal translucency measurements (>220) per year derived by second-order polynomial regression analysis. Three of the operators were certified by the Fetal Medicine Foundation (black), while the remaining three were not (grey). The reference curve of Nicolaides et al. [179] (dashed) is also shown.

The newly established curve was then used to recalculate the NT-MoM values for the entire study population; *Table 1* gives the observed median NT and 5th, 95th and 99th centiles for each 5-mm CRL interval, expressed both as absolute values and MoMs calculated using the new formula. There were no major differences in median NT-MoM values or 5th percentiles among the CRL intervals. Notably, 95th and 99th percentiles tended to be lower at higher CRL. The NT-MoM distribution resulting from this newly derived curve was used to model the combined screening performance (DR and FPR) that hypothetically would have been achieved in this population had the observed NT measurements matched the reference formula used for expected median NT. Data from 2005 and 2006 were analyzed separately. At a 5% FPR, the DR would have increased from 63 to 67% in 2005 and from 66 to 70% in 2006. A similar increase in screening performance is therefore expected to result from the standardization and quality control of NT measurements, as the observed distribution should more closely match that described by the reference formula used.

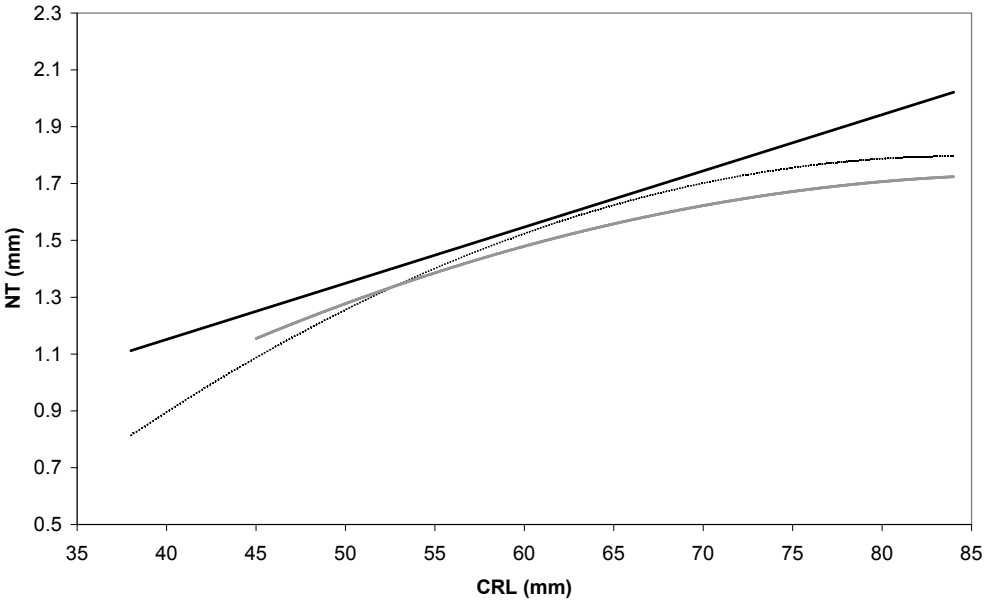

Figure 4 – Crown–rump length/nuchal translucency curve estimated for the Dutch population of operators (grey) using polynomial regression analysis. This curve was compared to the reference curves of both Nicolaides et al. [179] (black) and Spencer et al. [180] (dashed).

Discussion

In this study the quality of NT measurements in the Netherlands was analyzed by means of quantitative evaluation. According to quality demands cited by the Centre for Population Research, an operator must perform at least 150 NT measurements per year [175].

Only 22% of all operators met this demand in the studied period. From the viewpoint of prenatal screening for Down syndrome, median NT-MoM values should approximate to the target value of 1.0, with limited variation. Here, no relationship was found between the yearly number of NT measurements per operator and the median NT-MoM or the 5th–95th percentile range. Thus, the number of NT measurements per year does not seem to contribute to higher quality of these measurements.

In the analysis of the entire population of operators the mean median NT-MoM values of 0.94 (as compared to the Nicolaides *et al.* reference curve) and 0.98 (as compared to the Spencer *et al.* reference curve) were found. For Dutch operators, the latter curve seemed to give a more accurate estimate of expected medians. Over the period July 2002 to May 2004 a mean median NT-MoM of 0.83, based on the reference curve of Nicolaides *et al.*, was described [53]. In the current study, the median NT-MoM increased over time (*Figure 2*) and was 0.96 at the end of 2006. Growing awareness concerning quality control and education and training for operators may have contributed to this improvement. At the end of 2006 only 39% of the operators were FMF-certified to perform NT measurements. The CRL/NT curves of this group were generally closer to the published reference curves than those of non-certified operators. However, operators without FMF-certification are not by definition less educated as there are several other accredited NT courses in the Netherlands.

Based on all available NT measurements a new reference curve specifically tailored to our study population was calculated. The median NT-MoM values, recalculated with this new reference curve, were close to 1.0 for all intervals of CRL, and the overall downward trend in 95th and 99th percentiles, when expressed as MoMs, is in accordance with previously reported data [187]. The new curve therefore seems to accurately reflect the distribution of NT measurements in the study population, and was used to model the screening performance that could have been expected had the observed NT measurements matched the reference distribution.

The effect of using a reference distribution that matched the observed values on the Down syndrome screening performance appeared to be an increase of 4% in DR at a 5% FPR. However, this model was based on relatively few Down syndrome cases (n = 70). Therefore the distributions of the model parameters, especially NT-MoM, did not completely correspond to those of other, commonly used, models [188, 189]. This may explain the relatively low modelled DR.

In line with previous publications concerning the quality of NT measurements in the Netherlands [53, 177], it can be concluded that during the study period the average NT measurements were below the reference curves. Since 2007 NT measurements in the Netherlands have all been compared to the Spencer *et al.* CRL/NT reference curve, which in this study has been shown to be a better estimate for the Dutch population. Also, as from 2007, operators in the Netherlands are obliged to obtain certification from an accredited

organization. For these reasons it can be expected that Dutch operators will in future obtain median NT-MoMs closer to the target value and so, the screening performance across the country should improve. Continued qualitative and quantitative monitoring of NT measurements, however, remains important. Operators will be subject to annual reports, with median NT-MoM values, 5^{th}–95^{th} percentiles and CRL/NT curves calculated. Other quantitative and qualitative methods may also be applied [183, 190]. If necessary, operators will be given the chance to improve the quality of their NT measurements (e.g. by additional training), in order to further optimize the performance of the first-trimester combined test.

Acknowledgments

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Part I | Chapter 5

Performance of first-trimester serum screening for trisomy 21 before and from 11+0 weeks of gestational age in the Netherlands

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Abstract

In this paper the performance of first-trimester screening for Down syndrome (DS) before and from 11+0 weeks of gestation was evaluated in terms of detection rate (DR) and false positive rate. The study included 223 DS cases from the Dutch DS screening programme where maternal serum was collected between 8 and 14 weeks and the nuchal translucency between 11 and 14 weeks.

With the first-trimester combined test, 171 of the 223 DS cases were detected (DR = 77%). When serum collection took place before 11+0 weeks the DR was 84% (95%CI: 75-93). When serum collection took place at or after 11+0 weeks the DR was 73% (95% CI: 66-80). While the difference in DR between these two time periods was not statistically significant, we did recognize a tendency towards better screening performance early in the first trimester; the likelihood ratio for the 'early' group was 23 (95% CI: 20-28)) versus 16 (95% CI: 14-18) in the 'late' group.

Moreover, we found that PAPP-A performs best early (<11 weeks) and fβ-hCG later in the first trimester (≥11 weeks). Consequently, further improvement of the first-trimester combined test performance might be reached through serum collection at two different moments in pregnancy.

Down Syndrome (DS) is associated with increased maternal age, increased foetal nuchal translucency (NT), decreased maternal serum concentrations of PAPP-A and increased maternal serum concentrations of fβ-hCG. These parameters are all part of the first-trimester combined test which is a well established non-invasive method to identify pregnancies at risk for DS, and which is carried out routinely in several countries [54, 191, 192]. Maternal serum is collected between 8+0 and 13+6 weeks of gestational age (GA). The NT is measured at a crown-rump length (CRL) between 45 and 84 mm, which corresponds to 11+0 to 13+6 weeks of GA. The detection rate (DR) of the first-trimester combined test ranges from 64% to 87% with a 5% false-positive rate (FPR) [56]. In the Netherlands, first-trimester screening is performed since 2002 and recent reports show a DR of 76% for a FPR of 3.3% at a cut-off risk of 1 in 250 at term [54].

A recent study of Kirkegaard *et al.*, reported a first-trimester DR of 100% if serum was collected before 10 weeks of gestation compared to a DR of 77% when the serum was obtained at or after 10 weeks [193]. Here we present a similar study to investigate whether the timing of serum collection has an effect on the screening performance of the first-trimester combined test in the Netherlands, to possibly improve its performance.

The Dutch National Institute for Public Health and the Environment (RIVM) is the major DS screening centre in the Netherlands. Currently, the RIVM processes about 10,500 first-trimester combined screening tests per year. The GA at the time of serum collection (between 8+0 and 13+6 weeks) was determined either by ultrasound measurement or by first day of last menstrual period (LMP) depending on the preference of the applying health professional. PAPP-A and fβ-hCG concentrations were measured using an automated dissociation-enhanced lanthanide fluorescent immunoassay (AutoDelfia; PerkinElmer, Turku, Finland). Serum levels were expressed as maternal weight-corrected multiples of the gestation-specific normal median (MoMs).

All first-trimester screening results were registered in a database that contained information about maternal age and weight, GA, CRL, serum marker concentrations, NT and information about pregnancy outcomes (gestational age at birth, gender, birth weight and chromosomal disorders) reported through questionnaires by the participating women. The risk for DS was calculated using the software package LifeCycle (version 2.2, PerkinElmer, Turku, Finland). A pregnancy was classified as high risk when the calculated risk for DS was greater than or equal to 1 in 200 at the moment of risk assessment.

The database contained 223 pre- or postnatally diagnosed DS cases. Of all 223 cases, 171 were classified as high risk, resulting in a DR of 77% (95% CI: 71-82). To estimate the effect of the timing of serum collection on the screening performance the cases were divided in two groups (<11+0 weeks ('early' [*n* = 69]) and ≥11+0 weeks ('late' [*n* = 154])) based on the GA at serum collection. In the 'early' group, 58 out of 69 cases were classified as high risk for DS (DR 84% (95% CI: 75-93)). In the 'late' group a high risk was calculated for 113

out of 154 cases (DR 73% (95% CI: 66-80)). The difference between the DR of both groups was not statistically significant. Further analysis was performed to investigate whether the observed difference in DR could be caused by the timing of the NT measurement. However, the median CRL at the time of the NT measurement was not significantly different between the two groups (59.0 mm in the 'early' and 61.6 mm in the 'late' group; p = 0.228), which indicated that the timing of the NT measurement did not influence the DR.

Table 1 – Median MoMs of the first-trimester serum markers PAPP-A and fβ-hCG, detection rates (DR) at different cut-offs, and likelihood ratios (LR) for every gestational week. Because of the small number of cases week 8 and 9 were combined.

				DR (%) at cut-off risk		LR at cut-off risk
Week	*n*	fβ-hCG MoM	PAPP-A MoM	1 in 300	1 in 200	1 in 200
8+0 to 9+6	14	1.39	0.46	72	71	35
10+0 to 10+6	55	1.38	0.32	87	87	21
11+0 to 11+6	49	1.40	0.47	77	75	14
12+0 to 12+6	77	2.19	0.62	73	70	16
13+0 to 13+6	28	2.78	0.61	79	79	20
< 11 wk	69	1.38	0.38	84	84	23
≥ 11 wk	154	1.95	0.58	75	73	16

To evaluate the difference in FPR, a group of 27,068 unaffected, singleton pregnancies was selected and also divided into two groups based on the GA at the time of the serum collection. A significantly different FPR was found before 11 weeks of gestation (n = 4,911; FPR = 3.6% (95% CI: 3.1-4.1)) compared to at or after 11+0 weeks (n = 22,157; FPR = 4.6% (95% CI: 4.3-4.9)).

The likelihood ratio (LR) is an illustrative measure to give an indication of overall first-trimester screening performance, because it incorporates both DR and FPR. In our study the LR for the 'early' group (LR = 23 (95% CI: 20-28)) was significantly higher than that in the 'late' group (LR = 16 (95% CI: 14-18)).

To study a possible cause of the difference in screening performance, the MoM-distributions of PAPP-A and fβ-hCG were analysed throughout the first trimester. The range of MoMs in relation to GA in DS cases is shown in *Table 1* and *Figure 1*. As expected, the median PAPP-A MoM was decreased (0.50; $p < 0.0001$) and the median fβ-hCG MoM was increased (1.72; $p = 0.01$) in the DS cases. However when stratified, the PAPP-A MoM for DS was lower before 11 weeks of gestation (0.38 vs. 0.58) while for fβ-hCG MoM it was higher (1.95 vs. 1.38), and thus more distinctive from unaffected pregnancies at or after 11 weeks of gestation. After dividing our data into particular gestational weeks, the trend continued for both markers

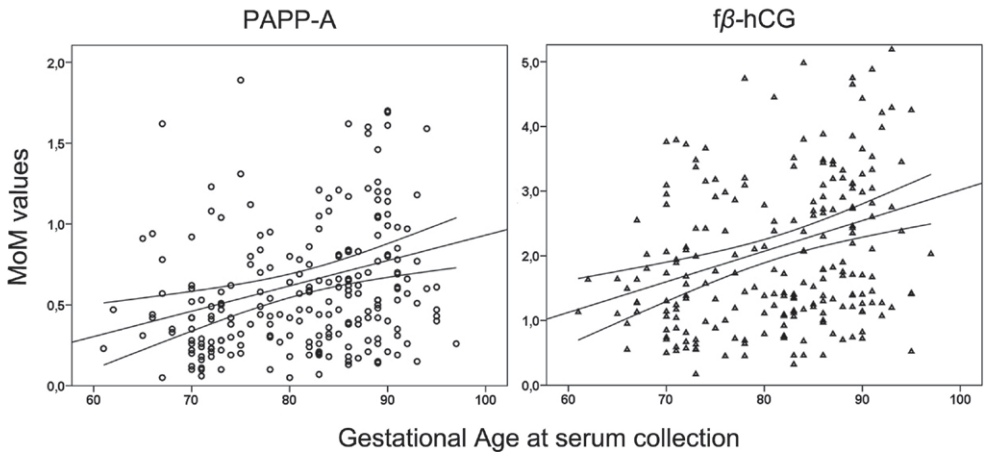

Figure 1 – Scatter plots and linear regression trend lines (95% CI) of MoM values of PAPP-A (circles) and fβ-hCG (triangles) in 223 Down syndrome cases.

except for the earliest measurement (8+0 to 9+6 weeks). This deviation may be explained by the low number of cases in this particular subgroup (n = 14). The correlations of MoM values of both markers with GA were highly significant in the DS cases (r = 0.231, p = 0.001 for PAPP-A and r = 0.278, p < 0.0001 for fβ-hCG). This trend of increasing MoMs during gestation was not found in the control pregnancies (data not shown).

We recognise the tendency of a higher DR with a lower FPR for the earlier compared to the later collection of maternal serum for first-trimester DS screening. Using the data of Kirkegaard *et al.* we calculated a LR < 10 wk of 29 (95% CI: 25-33) and a LR ≥ 10 wk of 18 (95% CI: 15-23). The LR of correctly predicting a DS pregnancy is, in both studies, significantly higher in the 'early gestation' group. However, in our current study we did not find a statistically significant difference between the DR of both groups.

This might be explained by differences in the study design of both studies. Firstly, in our study the GA cut-off between both groups was set on 11 weeks instead of 10 weeks of GA, because of the relatively small number of DS cases before the 10th week of gestation (n = 14). Nevertheless, the DR was highly comparable regardless the inclusion of week 10 (*Table 1*). Secondly, in the Netherlands the cut-off risk is 1 in 200 at the time of risk assessment, while in the Kirkegaard *et al.* study, it was 1 in 300. Therefore we analyzed our data at various cut-off risks, but this hardly influenced DR and FPR (*Table* 1) Thus, differences in cut-off of GA and of risks did not seem to underlie the between-study differences in DR. We rather presume that, although the numbers of DS cases in both studies were substantial, the study populations were too small to perform a proper statistical analysis of observed data without modelling.

In this study it was shown that in DS the MoMs of both first-trimester serum markers increase with GA. Combining our results with previously published meta-analyses, it turns out that, for DS screening purposes, PAPP-A performs best early in the first trimester and, oppositely, fβ-hCG later in the first trimester [193-197]. Therefore, we hypothesize that improvement of the screening performance could be reached through serum collection at two different moments in pregnancy; PAPP-A concentrations should be measured before the 11th week and fβ-hCG a few weeks later, e.g. at the same time as the NT measurement.

Part II | Chapter 6

Trisomy 18 and 13 screening with a simple algorithm: consequences for the Dutch Down syndrome screening programme

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Abstract

In this paper a prediction is made of the consequences for the Dutch Down syndrome screening programme in terms of detection rate (DR) and false positive rate (FPR) if trisomy 18 and 13 screening is introduced, using the algorithm and retrospective data of the Dutch programme, collected between 2004 and 2008.

MoM values (and 5^{th}-95^{th} percentiles) of PAPP-A, fβ-hCG and NT for trisomy 18 (n = 43) were 0.19 (0.06-0.98), 0.22 (0.08-1.40) and 1.93 (0.60-6.65) respectively. For trisomy 13 pregnancies (n = 20) they were 0.22 (0.07-1.84), 0.49 (0.21-1.37) and 2.08 (0.69-3.43) respectively. With the trisomy 21 algorithm, 23 trisomy 18 cases (DR = 50%) and 14 trisomy 13 cases (DR = 70%) were detected. While the trisomy 18 algorithm alone gave a reasonable DR for both trisomy 18 and trisomy 13 at an FPR of less than 1%, the combined trisomy 21 and 18 algorithms worked best producing a 77% DR for trisomy 18 and a 80% DR for trisomy 13 at only 0.2% extra FPR. Consequently, an algorithm for trisomy 18 significantly improves the DR for trisomy 18 and 13 within the Dutch Down syndrome screening programme, implying a cost-effective introduction.

Edwards syndrome (trisomy 18) and Patau syndrome (trisomy 13) are the second and third most common autosomal trisomies after Down syndrome (trisomy 21). Most infants with a trisomy 18 or 13 die in utero and the others within the first year of life. Especially in case of trisomy 13, there is an increased risk of severe and early onset pre-eclampsia [198]. To enable an early termination of trisomy 18 and 13 pregnancies, and to avoid maternal complications, screening for trisomy 18 and 13 seems a sensible option, especially since modelling has shown that current first-trimester screening can detect both chromosomal anomalies with a high detection rate (DR) and low false positive rate (FPR) [199, 200].

From January 2007 a governmentally approved national screening programme for Down syndrome has been implemented in the Netherlands, using the first-trimester combined test (pregnancy-associated plasma protein A [PAPP-A], the free beta subunit of human chorion gonadotropin [fβ-hCG] and nuchal translucency [NT]). The government license for this screening programme is strictly confined to screening for trisomy 21. Under the current license, it is not allowed to report on the risks for trisomy 18 and 13. However, since an increasing number of health care providers know the potential role of serum screening for trisomy 18 and 13, including some who already counsel their patient regarding these anomalies and since these trisomies are associated with early maternal complications it was recently decided to file a request to extend the government license to Edwards and Patau syndrome. This request is currently under review.

The aim of this article is to predict the consequences for the Dutch screening programme in terms of DR and FPR if trisomy 18 and 13 screening is introduced, using an accepted algorithm and retrospective data of the Dutch programme.

Trisomy 18 and 13 cases were selected from all first-trimester combined tests processed at our laboratory between 2004 and 2008 of which follow-up data was known. For all cases MoM values, a Down syndrome risk at test and an Edwards risk at test were calculated using LifeCycle2.2 risk calculation software (PerkinElmer, Turku, Finland). Distribution parameters for trisomy 18 were for PAPP-A: mean log MoM = −0.752 (120 cases) and SD log MoM = 0.38 (120 cases). For fβ-hCG they were: mean log MoM = −0.499 (247 cases) and SD log MoM = 0.40 (120 cases). The r-value for the correlation of log MoM PAPP-A and fβ-hCG was 0.061 (120 cases). The parameters were derived from an unpublished meta-analysis (source of data in use in Leeds in December 2002). For NT the mean log MoM was 0.442 (106 cases) and the SD log MoM was 0.27 (106 cases) [201]. The prior risk for trisomy 18 was 0.1 times the trisomy 21 risk. The foetal death rate was a fixed 17% for the first trimester. Thus, in the first trimester, the prior trisomy 18 risk was on average 0.37 ± 0.03 times that of trisomy 21. Of combined tests processed between April 2004 until December 2008, 43 trisomy 18 cases and 20 trisomy 13 cases were identified through self-reporting of pregnant women upon delivery. The median MoM values (and 5^{th}–95^{th} percentiles) of PAPP-A, fβ-hCG and NT for trisomy 18 were 0.19 (0.06–0.98), 0.22 (0.08–1.4) and 1.93 (0.6–6.65), respectively. For

trisomy 13 pregnancies, they were 0.22 (0.07–1.84), 0.49 (0.21–1.37) and 2.08 (0.69–3.43), respectively. The mean log MoM (±SD) for PAPP-A, fβ-hCG and NT for trisomy 18 were −0.65 ± 0.42, −0.66 ± 0.41 and 0.25 ± 0.36, respectively, and for trisomy 13 they were −0.57 ± 0.43, −0.28 ± 0.30 and 0.23 ± 0.25, respectively. For trisomy 18, the correlation coefficient of log MoM PAPP-A and fβ-hCG was 0.424 and for trisomy 13 it was 0.198.

Table 1 – Detection rate (DR) and corresponding 'high risk' test results (FPR) of trisomy 18 and 13 using a trisomy 21 algorithm, a trisomy 18 algorithm, or a combination.

	number of detected cases (DR)			
	trisomy 18 (n=43)	trisomy 13 (n=20)	trisomy 18 and 13 (n=63)	number of high risk results (FPR)
T18 algorithm cut-off ≤1:200	33 (77)	13 (65)	46 (73)	201 (0.9)
T18 algorithm cut-off ≤1:100	30 (70)	12 (60)	42 (67)	142 (0.6)
T18 algorithm cut-off ≤1:50	25 (58)	11 (55)	36 (57)	105 (0.5)
DS algorithm cut-off ≤1:200	23 (53)	14 (70)	37 (59)	992 (4.4)
DS algorithm cut-off ≤1:200 combined with T18 algorithm cut-off ≤1:200	33 (77)	16 (80)	49 (78)	1037 (4.6)
DS algorithm cut-off ≤1:200 combined with T18 algorithm cut-off ≤1:100	30 (70)	15 (75)	45 (71)	1009 (4.5)
DS algorithm cut-off ≤1:200 combined with T18 algorithm cut-off ≤1:50	25 (58)	14 (70)	39 (62)	995 (4.4)

Calculated risks represent the risk at test.

In the Netherlands the DR for trisomy 21 is 76% at a 3.4% FPR. DR for trisomy 18 and 13 were determined using either the algorithm for trisomy 21, the algorithm for trisomy 18 (at three cut-off risks) or a combination of the trisomy 21 and trisomy 18 algorithms. The corresponding FPR was determined using all first-trimester combined tests (n = 22,543) collected between April 2007 and December 2008 since for these tests also the trisomy 18 risk was calculated (but not reported to the pregnant women). Results are presented in *Table 1*. With the trisomy 21 algorithm, 23 (DR = 50%) trisomy 18 cases and 14 (DR = 70%) trisomy 13 cases were detected. While the trisomy 18 algorithm alone gave a reasonable DR for both trisomy 18 and trisomy 13 at an FPR of less than 1%, the combined trisomy 21 and 18 algorithms worked best producing a 77% DR for trisomy 18 and a 80% DR for trisomy 13 at only 0.2% extra FPR.

The median MoMs as presented in this study were quite comparable with those presented in the literature. A meta-analysis summarizing over 20 studies showed PAPP-A MoMs of

0.26 and 0.14, and fβ-hCG MoMs of 0.58 and 0.31 for trisomy 18 and 13, respectively [58, 202]. A more recent study also showed quite comparable PAPP-A MoMs of 0.2 and 0.3 and fβ-hCG MoMs of 0.2 and 0.5 for trisomy 18 and 13, respectively [203]. The mean log MoM and SD, and correlation coefficients were slightly different from the ones used in the applied algorithm. If analysis of additional trisomy 18 and 13 cases confirms this difference, it warrants adjusting the distribution parameters in the risk estimation software.

The DR and FPR in our population were slightly lower than those derived from modelled data [58]. This may in part be due to the fact that for pregnancies with a large NT the combined test was not completed. Within the Dutch programme, pregnancies with a NT exceeding 3.0 mm are eligible for extensive ultrasound examination, regardless of the results of the biochemical analysis. Moreover, at least during part of the study period, the median NT-MoM within the programme was reduced (0.89 MoM) [204]. Consequently, this could lead to an underestimation of the NT-MoM compared with the distribution parameters in use and thus an underestimation of the DR. To overcome this, we could establish distribution parameters based on our own data. However, currently there are too few cases to do so. Alternatively, we should pursue the inclusion of pregnancies with an NT that exceeds 3.0 mm.

Since there may be a difference in parental acceptance of a trisomy 21 pregnancy, as opposed to trisomy 18 or 13, or other pregnancy-related disorders like pre-eclampsia, it seems preferable to give individual risks for all of these disorders in the future. Especially, the distinction between the aneuploidies and pre-eclampsia seems relevant. Recently discovered screening markers, e.g. a disintegrin and metalloprotease 12 (ADAM12) [155] and placental growth factor (PlGF) [150], may be put to use for this. Sonographically measured foetal heart rate may be a good parameter to distinguish between trisomy 18 and 13 [203].

In conclusion, we have shown that an algorithm for trisomy 18 significantly improves the DR for trisomy 18 and 13 within the first-trimester screening programme for trisomy 21. Screening for trisomy 18 and 13 combines, therefore, considerable extra DR and a low extra FPR, and thus low additional financial costs. The cost of extra invasive testing and emotional costs in terms of extra maternal anxiety are obviously low as well.

Part II | Chapter 7

Placental protein 13 as a first-trimester screening marker for aneuploidy

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Abstract

Objective To determine whether placental protein 13 (PP13) could be an additional marker in first-trimester screening for aneuploidies.

Methods To evaluate differences in multiples of the gestation-specific normal median (MoMs), PP13 concentrations were measured in serum samples from Down syndrome, trisomy 18 and 13 affected pregnancies and euploid singleton pregnancies (four for each case matched for duration of storage, maternal weight and age).

Results The PP13 MoM in Down syndrome cases (n = 153) was 0.91 (not statistically significant from controls [n = 853]; $p = 0.06$; Wilcoxon rank sum test, two-tail). PP13 MoMs were decreased in trisomy 18 (n = 38; median MoM 0.64; $p < 0.0001$) and trisomy 13 cases (n = 23; median MoM 0.46; $p < 0.0001$).

There was a slight upward trend in MoMs of the Down syndrome cases with gestational weeks. The PP13 MoM was significantly correlated with the pregnancy-associated plasma protein A MoM and the free beta subunit of human chorion gonadotropin (fβ-hCG) MoM.

Conclusion PP13 does not seem to be a good marker for Down syndrome. PP13 MoMs are, however, significantly lower in trisomy 18 and 13 pregnancies. The addition of PP13 to the current screening test could be valuable for improving the discrimination of aneuploid from euploid pregnancies.

Introduction

Placental protein 13 (PP13) is one of the few known proteins predominantly produced by the syncytiotrophoblast [205, 206]. It is thought to play a major role in the implantation and modelling of the common foeto-maternal blood spaces through binding to proteins between placenta and endometrium [207, 208].

Recently, PP13 concentrations in maternal serum have been found to be decreased in pregnancies complicated by pre-eclampsia (PE) and/or small-for-dates foetuses [209-213]. It is hypothesized that the alteration of angiogenic factors found in PE could be a result of an impaired placental function [214]. It has also been described that there is abnormal placental development in trisomy 21 (Down syndrome) and, to greater extend in trisomy 18 and 13 [79]. Therefore current screening markers produced by the placenta, such as pregnancy-associated plasma protein A (PAPP-A), isoforms of human chorion gonadotropin (hCG) and inhibin, can be altered in these pregnancies. Since PP13 is also produced by the placenta, concentrations of this protein could also be altered in trisomic pregnancies. If so, PP13 might be an additional marker for aneuploidy screening.

To the best of our knowledge, this study on first-trimester PP13 concentrations in maternal serum of trisomy 21, 18 and 13 affected pregnancies is the first study in which the association between aneuploidies and PP13 is investigated.

Methods

Serum samples were collected at the National Institute for Public Health and the Environment (RIVM) between 2004 and 2006 as part of the Dutch national first-trimester Down syndrome screening programme. Samples were drawn between 8 and 14 weeks of gestational age and serum analysis of PAPP-A and the free beta subunit of hCG (fβ-hCG) was performed. For all requests maternal age, gestational age at sampling (GA), maternal weight and smoking status were recorded, as well as data on the nuchal translucency measurement. The health professionals who requested the test determined the gestational age at blood sampling and the method of calculation (either last menstrual period [LMP] or ultrasound dating). Women were asked to fill in a short questionnaire about the pregnancy outcome, including date of birth, birth weight, chromosomal abnormalities and pregnancy complications.

From this cohort, serum samples from Down syndrome, trisomy 18 and 13 pregnancies were selected and retrieved from storage. Four control sera from euploid singleton pregnancies were matched to each case, for the same day of gestation and as accurately as possible for sample date (± 6 months), maternal weight (within 5-kg weight class) and maternal age (± 2 years) at sampling. Baseline characteristics of the cases and controls are shown in *Table 1*.

Table 1 — Baseline characteristics in cases and controls.

	controls (*n*=853)*	Down syndrome (*n*=153)	Trisomy 18 (*n*=38)	Trisomy 13 (*n*=23)
Gestational age (days)†	81 (59-97)	81 (59-97)	82 (63-97)	78 (63-92)
Maternal weight (kg)†	67 (50-109)	67 (48-114)	68 (55-90)	67 (55-91)
Maternal age (years)†	37 (23-44)	37 (21-45)	38 (28-45)	37 (30-42)
Smoking (%)	64 (7.5)	13 (8.5)	6 (15.8)	2 (8.7)
Ethnicity (% Caucasian)	821 (96.2)	148 (96.7)	37 (97.4)	22 (95.7)

** Each case was separately matched to four control samples; three controls had to be excluded since there was not enough serum for analysis.*
† Presented as median values (range).

PP13 concentrations were measured using an automated dissociation-enhanced lanthanide fluorescent immunoassay (AutoDelfia; PerkinElmer, Turku, Finland).
PP13 levels were expressed in multiples of the gestation-specific normal median (MoMs). Normal medians were obtained by regression analysis in the controls of the median concentration for each completed week of gestation on the median days, weighted for the number of women tested. The observed MoM value was divided by the expected value for the maternal weight based on regression analysis in the controls of the median MoM on the median 1/weight in nine weight groups, weighted by the number of women. Furthermore, MoM values were divided by a correction factor for smoking and non-smoking women. Median MoM values and standard deviations of $\log_{10}$ transformed MoM values in cases and controls were estimated and statistically compared using the Wilcoxon rank sum test, two-tailed. Correlation coefficients were calculated for the associations of $\log_{10}$ PP13 with PAPP-A, fβ-hCG and gestation, after excluding outliers exceeding three standard deviations from the median. P-values <0.05 were considered statistically significant.

Results

Between 2004 and 2006 serum samples from 153 Down syndrome, 38 trisomy 18 and 23 trisomy 13 cases were collected. These samples, together with 853 control samples, were analyzed; three controls had to be excluded because there was not enough serum for analysis. *Figure 1* shows the distribution of PP13 concentrations in the controls. The regression equation for the normal median PP13 concentrations was: 10^(−0.316673 + 0.0494822 × GA – 0.000294144 × GA^2), where GA is the gestational age in days.
PP13 MoM values were significantly negatively related to maternal weight ($p < 0.005$). The regression equation for the expected MoM for a given weight was: 0.42293 + 40.1339/ weight.

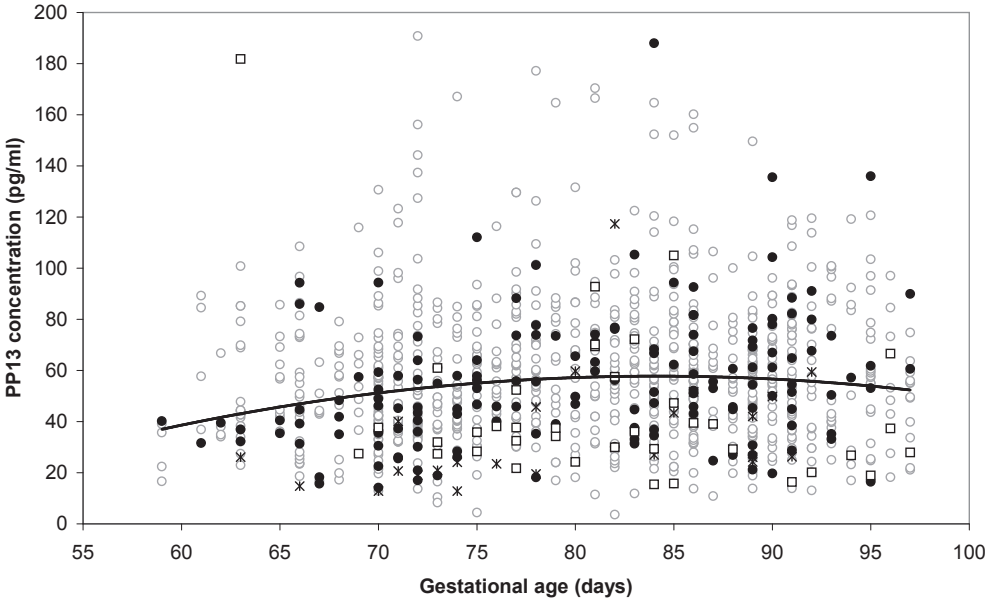

Figure 1 – Distribution of PP13 concentrations in Down syndrome (black dots), trisomy 18 (open squares), trisomy 13 (crosses) and unaffected pregnancies (open dots). The trendline represents a log-quadratic regression of the unaffected pregnancies.

Table 2 shows the median PP13 MoM values and standard deviations for cases and controls according to maternal smoking status. Smoking status was stated here as a dichotomous variable; no reliable quantitative information was available. For each type of case and for controls the median is lower in smokers, which was highly statistically significant among Down syndrome cases and controls (both $p < 0.0001$; Wilcoxon rank sum test, two-tailed). Correction for smoking was performed using the following equations: PP13 MoM/1.016 for non-smokers and PP13 MoM/0.635 for smokers. The dataset contained too few non-Caucasian women to study differences between ethnic groups.

Table 3 shows the median PP13 MoM values and standard deviations after correction for maternal weight and smoking in Down syndrome, trisomy 18 and 13 cases compared to controls. PP13 MoM levels were reduced on average in the three types of aneuploidy studied. In Down syndrome cases the reduction, with a median of 0.91 MoM, was not statistically significant ($p = 0.06$). However, there were highly significantly lower PP13 MoMs for trisomy 18 cases (0.64; $p < 0.0001$) and trisomy 13 cases (0.46; $p < 0.0001$).

Among the Down syndrome cases there was a statistically significant tendency for PP13 MoM values to increase with gestation in days (GA), with correlation coefficient 0.21 ($0.321 + 0.00841 \times GA$; $p < 0.01$). Similarly, in trisomy 13 where the r-value was 0.44 ($10^{(-1.09 + 0.0106 \times GA)}$; $p < 0.05$), whilst in trisomy 18 the trend was downwards (r = −0.31; 31.7 −

$0.728 \times GA + 0.00425 \times GA^2$; $p = 0.06$). PP13 was significantly correlated with both PAPP-A and fβ-hCG concentrations (*Table 4*). Correlation coefficients tended to be higher among the trisomic pregnancies.

Table 2 – Median MoM values and log_{10} MoM standard deviations (SD) in Down syndrome (DS), trisomy 18 (T18), trisomy 13 (T13) and unaffected pregnancies (controls) according to smoking status.

	Smoking	*n*	Median PP13 MoM	Mean log_{10} PP13 MoM	SD log_{10} PP13 MoM
DS	No	140	0.93	-0.019	0.177
DS	Yes	13	0.42	-0.315	0.203
T18	No	32	0.63	-0.163	0.242
T18	Yes	6	0.50	-0.266	0.126
T13	No	21	0.46	-0.240	0.184
T13	Yes	2	0.35	-0.490	0.124
Controls	No	789	1.02	-0.005	0.183
Controls	Yes	64	0.63	-0.201	0.207

Table 3 – Median MoM values and log_{10} MoM standard deviations (SD) in Down syndrome (DS), trisomy 18 (T18), trisomy 13 (T13) and unaffected pregnancies (controls) after correction for smoking.

	n	Median PP13 MoM	Mean log_{10} PP13 MoM	SD log_{10} PP13 MoM	*p*-value
DS	153	0.91	-0.033	0.175	0.0645
T18	38	0.64	-0.154	0.242	<0.0001
T13	23	0.46	-0.259	0.184	<0.0001
Controls	853	1.00	-0.011	0.186	-

Table 4 – Correlation coefficients of PP13 with PAPP-A and fβ-hCG in Down syndrome (DS), trisomy 18 (T18), trisomy 13 (T13) and unaffected pregnancies (controls).

	DS	*p*	T18	*p*	T13	*p*	Controls	*p*
PAPP-A	0.258	<0.01	0.658	<0.001	0.295	0.18	0.283	<0.001
fβ-hCG	0.288	<0.001	0.383	<0.01	0.532	<0.05	0.235	<0.001

Discussion

To our knowledge, this is the first report on PP13 as a potential screening marker for common aneuploidies. A case–control study was conducted using sera from Down syndrome, trisomy 18 and 13 affected pregnancies and matched controls.

The small decrease of PP13 MoM values in Down syndrome pregnancies was not statistically significant and PP13 is, therefore, not likely to greatly improve first-trimester screening for Down syndrome. However, PP13 MoM values were highly significantly decreased in trisomy

18 and 13 pregnancies. In our laboratory approximately 80% of all trisomy 18 and 13 cases are detected using a specific first-trimester algorithm, implying the importance of additional markers such as PP13 to improve performance. In the Netherlands, there is currently no screening programme for trisomies other than Down syndrome but implementation in the near future is foreseen.

There are a few specific findings that truly characterize a trisomic placenta. In Down syndrome pregnancies a decrease or delay in syncytial formation and morphological differentiation is present [77, 78]. At term, the placenta is considerably smaller in Down syndrome affected pregnancies compared to unaffected pregnancies [215], which might already be present in the first trimester. Furthermore, undervascularization and hypotrophy of the placenta have been described [80]. There is an extremely wide range in the extent of these effects of Down syndrome on placental development. In trisomy 18 and 13 these effects tend to be much larger. In trisomy 18 pregnancies the placental cell proliferation rate is increased [86]. It is possible that this increase in cell proliferation may actually be the result of increased cell death. The number of foetal capillaries per villus cross-section is reduced and this finding may offer an explanation for the early onset intrauterine growth restriction which characterizes this chromosomal abnormality [84, 216].

The trophoblast is the major source of placental specific hormones and proteins such as PP13. Since the highly polarized syncytiotrophoblast secretes its hormonal products into the maternal circulation with almost no storage capacity, any alteration in syncytiotrophoblast formation should be reflected in the maternal circulation.

Maternal smoking impairs placental development by changing the balance between cytotrophoblast proliferation and differentiation [217]. This may explain the lower PP13 concentrations in smoking compared to non-smoking women, an effect that has also been described by others [218]. Therefore, correction for smoking is of importance in a screening test containing placental markers like PP13. Since in this study the method of gestational dating was unknown the distribution parameters could not be calculated for LMP and scan dating separately. In the study period dating was more consistent with ultrasound dating [219]. According to Dutch policy ultrasound dating will be the method for gestational dating in the future and thus, the distribution parameters presented in here will be fitting for the future Dutch screening programme.

PP13 was found to be significantly correlated with fβ-hCG and to greater extend with PAPP-A. In the trisomy 18 and 13 cases these correlations were slightly higher compared to controls and sometimes the correlation coefficient was even larger than 0.5. High correlations between markers can be a sign of redundancy, however, this is not necessarily true [60]. Extensive modelling of all relevant first-trimester screening markers, taking into account their mutual correlations, will indicate the true predictive value of PP13 as a screening marker (to be published elsewhere).

Previous publications have shown that low first-trimester PP13 values are predictive of early PE [211, 212]. In this study, it was found that PP13 levels are significantly lower in trisomy 18 and 13 pregnancies. In these trisomies serum levels of PAPP-A and fβ-hCG are also largely decreased [220] which is not necessarily the case in pregnancies complicated by PE. Therefore, addition of PP13 to the current screening test could be valuable to make a proper distinction between normal, aneuploid and PE pregnancies. However, new algorithms would be needed to clinically implement such a screening program.

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Part II | Chapter 8

Modelling the Down syndrome screening performance using first-trimester serum markers

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Submitted

Abstract

Objective To evaluate the predictive value of three current (PAPP-A, fβ-hCG and NT) and four potential screening markers (ADAM12, thCG, PP13 and PlGF) for Down syndrome (DS), in combination with different screening settings.

Methods All markers were measured in stored first-trimester serum of 151 DS cases and 847 controls. All marker levels were expressed as multiples of the median (MoMs) and compared using a Mann-Whitney U test. Model predicted detection rates (DR) for fixed false-positive rates (FPR) were obtained.

Results Significantly different median MoMs for DS cases compared to controls were found for PAPP-A (0.49 vs. 1.00; $p < 0.0001$), fβ-hCG (1.70 vs. 1.01; $p < 0.0001$), ADAM12 (0.89 vs. 1.00; $p < 0.0001$), thCG (1.28 vs. 1.00; $p < 0.0001$), PlGF (0.80 vs. 1.00; $p < 0.0001$) and NT (1.74 vs. 1.01; $p < 0.0001$). The lower PP13 MoM in DS cases (0.91 vs. 1.00) was not statistically significant ($p = 0.061$).

Adding the four new markers to the current screening led to an increase in DR from 77% to 80% at a 5% FPR. The application of a two-sample screening model (some markers early and others later in the first-trimester) increased the DR to 89%. In a two-step contingent model with an intermediate cut-off range of 100-2000 after biochemical screening the overall DR was 77%, but only 33% of women had to be referred for a NT measurement.

Conclusion First-trimester DS screening may well be improved by adding new markers to the current screening test and by applying different screening settings. Application of a two-sample screening model resulted in the highest detection rate.

Introduction

In the Netherlands, all pregnant women are offered prenatal screening for Down syndrome (DS) by means of the first-trimester combined test. This test is composed of the maternal serum parameters pregnancy-associated plasma protein A (PAPP-A) and the free beta subunit of human chorionic gonadotropin (fβ-hCG) and a nuchal translucency (NT) measurement (sonographic marker) combined with maternal age. Based on these parameters a risk of having a child with DS is calculated and reported to the pregnant woman. This way, 76% of all DS cases are detected in the Dutch population at a 3% false positive rate (FPR) [54]. This percentage is rather low and indicates that there is a need for improvement.

Recently, several studies have been performed in which new potential DS screening markers were individually measured. A disintegrin and metalloprotease 12 (ADAM12), placental protein 13 (PP13) and placental growth factor (PlGF) have been shown to be decreased in DS pregnancies [150, 155, 221-223]. Moreover, total hCG (thCG), which is a screening marker for DS in the second trimester of pregnancy [224], has also been reported to be increased in first-trimester DS pregnancies [225]. Adding these markers to the current first-trimester combined test might provide higher detection rates (DR).

Additionally, these rates can be improved by the use of different first-trimester screening settings. Some markers are known to be more distinctive between DS cases and controls, respectively early or later in the first trimester [155, 197, 221, 223]. Based on this knowledge, it may be appropriate to draw two separate blood samples in the first trimester and then integrate them for the combined test (two-sample combined test).

In a first-trimester contingent screening setting only women with a risk above a certain cut-off, as determined by the biochemical markers, are offered further testing (i.e. NT measurement). At the same time, women with a very high risk as determined by biochemical testing could be offered an invasive diagnostic procedure directly, without further testing [226].

In this study, three current (PAPP-A, fβ-hCG and NT) and four potential DS screening markers (ADAM12, thCG, PP13 and PlGF) were used to model the first-trimester screening performance in combination with different screening settings to investigate how to improve detection rates.

Methods

Sample selection

Serum samples were collected at the Dutch National Institute for Public Health and the Environment (RIVM) as part of the Dutch first-trimester screening programme. Maternal blood samples were drawn between 8+0 and 13+6 weeks of gestation. After analysis, serum samples were stored at -30°C. Each sample was accompanied by a form containing information on maternal age, gestational age, maternal weight, number of foetuses, NT

and crown-rump length (CRL) and smoking status. Data on the NT measurement was not always available to our laboratory, because some applicants performed a combined risk calculation on-site. To the best of our ability, missing NT data was retrieved for the DS cases. Pregnancy outcome (chromosomal disorders, date of birth, birth weight) was recorded by questionnaires and collected through self-reporting of the participating women.

All reported Down syndrome (DS) cases between 2004 and 2006 (n = 151) were selected for this study. Controls were sampled from all singleton pregnancies of which information about the pregnancy outcome was known. Controls were matched to the cases by gestational age (exact day), maternal weight (± 5 kg), maternal age (± 2 years) and storage time (± 6 months). Two-tailed Mann-Whitney U tests and Pearson's chi-square tests were used to compare the baseline characteristics.

Serum concentrations of PAPP-A and fβ-hCG were measured as part of the first-trimester combined test using a semi-automatically performed time-resolved immunofluorometric assay (AutoDelfia; PerkinElmer, Turku, Finland). For this study, all samples were retrieved from storage and the other biochemical parameters were measured retrospectively, using either an AutoDelfia (ADAM12, thCG and PP13) or DelfiaXpress (PlGF) assay. Serum analysis was blinded for the diagnostic outcome.

Table 1 – Baseline characteristics of Down syndrome (DS) cases and controls in this study expressed as medians (inter-quartile range) or n (%). P-values were calculated using Mann-Whitney U tests or Pearson's chi-square tests.

	DS	Controls	*p*-value
n	151	847	
Gestational age (days)	81 (72-88)	81 (73-88)	0.668
Maternal age (years)	37 (36-39)	37 (36-39)	0.050
Maternal weight (kg)	67 (62-74)	67 (62-74)	0.531
Smoking	13 (8.6)	63 (7.4)	0.617
NT available	126 (83.4)	539 (63.6)	<0.001

Data analysis

Serum marker levels were expressed as multiples of the gestation-specific normal median (MoMs). Normal medians were obtained by regression analysis of the median concentration for each completed gestational week in the controls, weighted for the number of women tested. The observed MoM value was divided by the expected value for maternal weight based on regression analysis of the controls. When MoMs were significantly different between smoking and non-smoking women a correction factor for smoking was applied. Median MoMs in cases and controls were calculated and then statistically compared

using a Mann-Whitney U test (two-tailed). Correlation coefficients for all markers were calculated using the $\log_{10}$ concentrations and gestation, after excluding outliers exceeding three standard deviations from the median. P-values <0.05 were considered statistically significant.

Modelling

Model predicted detection rates (DR) for fixed false positive rates (FPR) were obtained for each marker and different combinations of markers by numerical integration. This assumed multivariate log Gaussian distributions fit both DS and unaffected pregnancies. The theoretical range of MoMs was divided into a number of equal sections thus forming a 'grid' in multi-dimensional space. The Gaussian distributions were then used to calculate for each section (square for two markers, cube for three etc): the proportion of DS and unaffected pregnancies in the section and the likelihood ratio (LR) between them. The appropriate centiles of LR (95th, 97th and 99th centile respectively) in unaffected pregnancies were determined and the proportion of DS pregnancies with these values or higher was the predicted DR. The model parameters were the observed $\log_{10}$ medians, $\log_{10}$ standard deviations and Pearson's correlation coefficients. A standard age distribution with mean = 27 years and standard deviation (SD) = 5.5 years was used [227]. This way DR could be calculated for different marker combinations and screening settings (one-sample, two-sample and contingent). Correlations between biochemical markers in the two-sample screening model were assumed to be similar to those in the one-sample screening model.

Data were analyzed using the statistical software package SAS (SAS Institute, Cary, NC, USA).

Results

In *Table 1* the baseline characteristics of all DS cases and controls are shown. Controls were matched to the cases by gestational age, maternal age and maternal weight, so no significant difference would be expected. A highly statistically significant difference was found for the percentage of NT measurements available. This difference is due to the recall of missing NT measurements in the DS group as described in the methods section. Since we did not dispose complete data on ethnicity, we could not compare this.

Table 2 shows the overall median MoMs of all markers in DS cases and controls. Except for PP13, a statistically significant difference between groups was found for all markers. Furthermore, median MoMs were calculated for every gestational week. Interestingly, some biochemical markers showed a more distinctive ratio between DS cases and controls early in the first trimester (PAPP-A, ADAM12 and PP13) while others were more distinctive later in the first trimester (fβ-hCG, thCG and PlGF). *Table 3* shows the correlation coefficients between the markers.

Table 2 – Median MoM values of all markers in this study by gestational week in Down syndrome (DS) cases and controls (con). MoMs were also calculated for an 'early' first-trimester week group (8-10 weeks) and a 'late' first-trimester week group (11-13 weeks). Log_{10} standard deviations (SD) are given for the entire population. P-values were calculated using a Mann-Whitney U test.

	n		PAPP-A		fβ-hCG		ADAM12		thCG		PP13		PlGF		NT *	
week	DS	con	DS	con	DS	con	DS	con	DS	con	DS	con	DS	con	DS	con
8	3	12	0.49	0.93	1.16	1.06	0.45	1.07	0.75	0.97	0.91	1.11	0.86	1.08	-	-
9	18	81	0.52	1.03	1.50	1.15	0.77	1.01	1.07	1.10	0.84	1.05	0.85	0.98	-	-
10	39	216	0.36	0.98	1.70	0.97	0.72	1.00	1.12	0.95	0.84	0.95	0.80	1.00	-	-
11	27	181	0.44	1.00	1.43	0.98	0.98	0.96	1.36	0.93	0.94	1.03	0.92	1.01	2.04	0.97
12	42	237	0.59	1.02	2.17	0.98	1.01	1.02	1.62	1.00	0.93	0.98	0.72	0.99	1.62	1.02
13	22	120	0.58	0.99	2.48	1.05	1.14	0.98	1.90	1.03	1.08	1.01	0.78	0.99	1.64	1.00
8-10	60	309	0.42	0.99	1.63	1.03	0.73	1.01	1.09	1.00	0.84	1.00	0.84	0.99	-	-
11-13	91	538	0.57	1.00	2.03	0.99	1.02	1.00	1.56	1.00	1.01	1.00	0.78	1.00	1.74	1.01
Total median	151	847	0.49	1.00	1.70	1.01	0.89	1.00	1.28	1.00	0.91	1.00	0.80	1.00	1.74	1.01
Total SD	151	847	0.31	0.25	0.28	0.27	0.19	0.16	0.20	0.19	0.17	0.19	0.14	0.14	0.23	0.13
p-value			<0.001		<0.001		<0.001		<0.001		0.06		<0.001		<0.001	

** Data on the NT measurement was not always available; therefore the number of cases and controls does not apply for the NT measurement.*

To analyze the predictive value of all markers several models were constructed. For the one-sample screening setting overall median MoMs were used to calculate the DR for given FPRs of 5%, 3% and 1% respectively (*Table 4*). In this setting, none of the new potential markers on itself added substantially to the current screening markers (PAPP-A, fβ-hCG and NT). Adding all markers to the current screening model yielded a 3-4% increase in DR. Reducing the number of markers in the one-sample model by backward selection successively led to the exclusion of PP13 and thCG. Without these markers the DR was 79% at a 5% FPR, which was only 1% lower than the model with all seven markers.

To simulate a two-sample screening setting within the first trimester, two week groups were created (8-10 weeks and 11-13 weeks respectively). In the two-sample screening model statistical parameters of the 'early' week group were used for PAPP-A, ADAM12 and PP13 and parameters of the 'late' week group were used for fβ-hCG, thCG, PlGF. Obviously, since the NT measurement takes place between 11-13 weeks, the statistical parameters of this marker were equal to those in the one-sample screening model. Again, DR was calculated for different marker combinations (*Table 4*). Using a two-sample screening model the DR increased from 77% to 83% at a fixed 5% FPR. When adding the four new markers to this model the DR increased even further to 89%.

Table 3 – Pearson's correlations coefficients between all biochemical markers in Down syndrome (DS) cases and controls.

DS	PAPP-A	fβ-hCG	ADAM12	thCG	PP13	PLGF
PAPP-A	1.000					
fβ-hCG	0.191	1.000				
ADAM12	0.460	0.297	1.000			
thCG	0.182	0.715	0.598	1.000		
PP13	0.408	0.389	0.528	0.627	1.000	
PlGF	0.152	-0.124	0.154	-0.022	0.018	1.000

Controls	PAPP-A	fβ-hCG	ADAM12	thCG	PP13	PLGF
PAPP-A	1.000					
fβ-hCG	0.186	1.000				
ADAM12	0.413	0.152	1.000			
thCG	0.221	0.677	0.434	1.000		
PP13	0.324	0.287	0.432	0.531	1.000	
PlGF	0.256	0.102	0.324	0.189	0.216	1.000

All correlations with NT were assumed zero.

In the first step of the contingent screening model shown in *Table 5* all biochemical markers were analyzed to calculate an intermediate risk for DS. This way, 38-60% of all DS cases could be detected early, depending on the low risk and high risk cut-off values. The early assurance after the first contingent step varied from 67-90%, indicating that only a small percentage of women needs to continue the screening procedure with a second contingent step (i.e. NT measurement). With this model, and using all markers, an overall DR of 77% at a given 5% FPR could be achieved at an intermediate risk cut-off of 100-2000, which is equal to the overall DR of the current markers in a one-sample screening model (*Table 4*).

Discussion

Compared to international studies [48, 55, 56], the detection of Down syndrome in the Dutch population is rather low [53, 54] and thus allows for improvement. The aim of this population-based study was to evaluate the predictive value of four new potential first-trimester screening markers for DS and to examine in which screening setting they would be of best use.

We found a statistically significant difference in MoMs between DS and control pregnancies for ADAM12, thCG and PlGF, but none of these markers showed a significant improvement in DR when added to the current one-sample screening model with PAPP-A, fβ-hCG and NT. The joint addition of all new markers led to an improvement in DR of only 3-4%, which is disappointing considering that four extra markers had been added. However, with newly developed techniques that facilitate the analysis of multiple markers simultaneously (i.e. bead-based immunoassays or antibody-arrays) a multi-marker model for DS screening might be cost-effective. It should be taken into account though, that constructing multi-marker models always involves a risk of over-fitting [228]. Therefore it could be argued that the one-sample model in this study would be better without PP13 (non-significant difference in MoMs) and thCG (highly correlated with fβ-hCG) which would only result in a 1% lower detection rate (*Table 4*).

In this study we found markers which were more distinctive between DS cases and controls early in the first trimester, while others performed better later in the first trimester. The application of a two-sample screening setting on the current DS screening markers (‘early’ PAPP-A; ‘late’ fβ-hCG) would already result in a 6-7% higher DR. When the remaining four screening markers were added to the model (‘early’ ADAM12 and PP13; ‘late’ thCG and PlGF) the DR increased with another 6-7%. Strikingly, unlike in the one-sample screening setting, the addition of thCG did significantly contribute to the DR of the two-sample screening setting. Adopting such a screening setting would require two blood samples to be withdrawn in the first trimester, which obviously bears extra costs, as does the use of extra screening markers. On the other hand, the benefits of extra DR are high and with the

disposal of multi-marker panels it would be possible to analyze all markers simultaneously and thus faster and cheaper. Moreover, if all markers would be measured at two times within the first trimester a "repeated measures approach" could also be applied [229] to improve the DR even more. With such a policy the first blood sample should be taken at 8-10 weeks and the second at the time of the NT measurement (11-13 weeks). For this modelling study we did not actually dispose of two serum samples from the same women and correlations between markers were assumed to be equal in both the one-sample as well as the two-sample screening model. However, the r-value could be smaller when markers are measured in different samples some weeks apart and DRs might therefore be even higher in the two-sample model.

Table 4 – Modelled detection rates (DR) at given false positive rates (FPR) for NT at 11-13 weeks and several serum marker combinations in a one-sample or two-sample test (models containing all markers are displayed in bold).

One-sample test	DR at FPR		
(8-13 wks)	5%	3%	1%
PAPP-A, fβ-hCG & NT	77	71	59
+ADAM12	77	72	60
+thCG	77	71	60
+PP13	77	71	60
+PlGF	78	73	61
+ADAM12, thCG, PP13 & PlGF	**80**	**74**	**63**
+ADAM12, thCG & PlGF	79	74	62
+ADAM12 & PlGF	79	73	62

Two-sample test		DR at FPR		
1st sample (8-10 wks)	2nd sample (11-13 wks)	5%	3%	1%
PAPP-A	fβ-hCG & NT	83	79	68
+ADAM12		84	80	69
	+thCG	85	80	70
+PP13		84	79	69
	+PlGF	85	80	69
+ADAM12 & PP13	**+thCG & PlGF**	**89**	**85**	**75**
+ADAM12	+thCG & PLGF	88	84	74
+ADAM12	+PlGF	85	81	70

In this study a two-step contingent screening setting, in which all biochemical markers were measured as a first step before NT measurement, was also simulated. With contingent screening, the overall performance will always be slightly lower compared to current combined screening because of the intermediate risk cut-off [226, 230]. However, we found that when all biochemical markers examined here would be added to the contingent model, the DR was similar to a one-sample model with the three current screening markers. The advantage of contingent screening is that only a small amount of women continues to the second contingent step. For example, when an intermediate risk cut-off of 100-2000 was used in this study, only one third of all women would be referred for a NT measurement.

Table 5 – Contingent screening model at different high risk and low risk cut-off values. In the first contingent step, all biochemical markers (PAPP-A, fβ-hCG, ADAM12, thCG, PP13 and PlGF) were analyzed leading to an early detection rate (DR), an early false positive rate (FPR) and early assurance. If necessary, a NT measurement was performed in the second contingent step leading to an overall DR.

high risk cut-off	low risk cut-off	early DR (%)	early FPR (%)	early assurance (%)	overall DR at 5% FPR
1 in 50	1 in 500	38	0.8	90	70
	1 in 1000	38	0.8	81	75
	1 in 2000	38	0.8	67	77
1 in 100	1 in 500	48	1.8	90	70
	1 in 1000	48	1.8	81	75
	1 in 2000	48	1.8	67	77
1 in 200	1 in 500	60	4.0	90	70
	1 in 1000	60	4.0	81	74
	1 in 2000	60	4.0	67	76

The NT measurement is considered to be a good, albeit difficult-to-measure, parameter demanding rigorous monitoring of the quality of this measurement [176, 183]. Performing less NT measurements, as in a contingent setting, could therefore reduce the costs of a screening programme [231].

To provide a proper comparison with previously published modelling studies a standard age distribution (mean = 27 years; SD = 5.5 years) was used for all constructed models in our study [227]. The mean maternal age in the Dutch screening population is actually higher (34 years) [54]. Since DR and FPR depend on the maternal age distribution the modelled DRs in this study are likely to be underrated, considering the relatively high maternal age of the Dutch population.

Based on the results of this study we come up with three possibilities to improve the first-trimester screening performance: 1) A one-sample combined screening setting with

four biochemical screening markers (PAPP-A, fβ-hCG, ADAM12 and PlGF) and the NT measurement, leading to minimal improvement of DR at low extra costs. 2) A two-sample combined screening setting with or without new screening markers, leading to a considerable improvement of DR, but probably at higher costs and a more complex organization due to a second blood withdrawal. 3) A contingent screening setting with as a first step six biochemical markers (PAPP-A, fβ-hCG, ADAM12, PlGF, PP13 and thCG) and as a second step the NT measurement, not leading to an improvement of DR, but to a reduction of costs due to less NT measurements.

In conclusion, we showed that, by adding new markers to the current first-trimester screening test and by applying different screening settings, first-trimester DS screening may well be improved. To improve the rather low DR of the Dutch population it would be advisable, pending a thorough cost-effectiveness analysis, to adopt a two-sample screening model.

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Part II | Chapter 9

Distributions of current and new first-trimester Down syndrome screening markers in twin pregnancies

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Abstract

Objective To study the distributions of pregnancy-associated plasma protein A (PAPP-A), the free beta subunit of human chorion gonadotropin (fβ-hCG), a disintegrin and metalloprotease 12 (ADAM12) and placental protein 13 (PP13) in first-trimester twin pregnancies.

Methods Serum marker concentrations were measured in monochorionic and dichorionic twin pregnancies and singleton controls to study differences in multiples of the gestation-specific normal medians (MoMs).

Results Median PAPP-A and fβ-hCG MoMs were 2.03 and 1.87 for monochorionic twins (n = 116) and 2.18 and 1.89 for dichorionic twins (n = 650). Furthermore, ADAM12 and PP13 MoMs were 1.66 and 1.56 for monochorionic twins (n = 51) and 1.64 and 1.53 for dichorionic twins (n = 249). No statistically significant differences between monochorionic and dichorionic twin pregnancies were found. Correlations between markers in these pregnancies did not differ from singletons.

Conclusion For first-trimester screening, different parameters for monochorionic and dichorionic twin pregnancies are not necessary. Furthermore, if ADAM12 and PP13 will be adopted as screening markers, the presented median MoM values, standard deviations and correlation coefficients for twin pregnancies may contribute to a proper twin risk estimation.

Introduction

The first-trimester combined test for singleton pregnancies to calculate the risk for Down syndrome is composed of the maternal serum concentrations of pregnancy-associated plasma protein A (PAPP-A) and the free beta subunit of human chorion gonadotropin (fβ-hCG), combined with the measurement of the nuchal translucency (NT). Because of the association between maternal age and the incidence of both Down syndrome and twin pregnancies, high-risk test results are more common in twin pregnancies [232]. Furthermore, invasive diagnostic tests, such as amniocentesis, are more difficult to perform in twin pregnancies. For these reasons, screening for Down syndrome in twin pregnancies is advisable, although complicated by several factors. Firstly, the risk estimation depends on zygosity, which is not always definable. Secondly, biochemical markers give information about the whole pregnancy and not on the individual foetus. Finally, due to the low number of Down syndrome cases in twin pregnancies, an unambiguous distribution of serum markers is not available and less accurate modelling techniques have to be applied.

It is important to determine chorionicity in twins. Chorionicity is an indication for the type of placentation. Monozygotic twins will have a monochorionic placenta in about 75% of cases [233]. All dizygotic twins will have a dichorionic placenta. Monochorionic twins share a placenta, while dichorionic twins each have their own. To distinguish between the two, ultrasound examination can be used [234, 235].

Despite many studies on the risk estimation for Down syndrome in twin pregnancies it remains unclear whether and to what extent there is a significant difference in biochemical parameter concentrations between monochorionic and dichorionic twins [236-242]. Previous studies of first-trimester serum screening concentrated mainly on determining normal values for PAPP-A and fβ-hCG in twin pregnancies [46, 239, 243, 244]. Recently, several new screening markers have been tested and their potential improvement concerning Down syndrome screening has been investigated [155, 223, 245, 246].

In this study, distributions of two new potential screening parameters (a disintegrin and metalloprotease 12 [ADAM12] and placental protein 13 [PP13]) in twin pregnancies were determined for the first time. Additionally, the distributions of the serum concentrations of the current first-trimester screening parameters (PAPP-A and fβ-hCG) in twin pregnancies were investigated. Moreover, it was studied whether a distinction between monochorionic and dichorionic twin pregnancies is necessary.

Methods

Serum samples were collected at the National Institute for Public Health and the Environment (RIVM) between 2004 and 2008 as part of the Dutch national first-trimester Down syndrome screening programme. Samples were drawn between 8 and 14 weeks of gestational age,

and serum analysis of PAPP-A and the fβ-hCG was performed. This cohort contained serum of monochorionic twin pregnancies (n = 116) as well as dichorionic pregnancies (n = 650). Chorionicity was determined by the health professional responsible for the test application. Mann–Whitney U tests and Pearson's chi-square tests were used to compare the baseline characteristics of both groups.

Serum samples from a selection of these twin pregnancies (51 monochorionic and 249 dichorionic twin pregnancies) were randomly retrieved from storage to measure concentrations of ADAM12 and PP13, using an automated dissociation-enhanced lanthanide fluorescent immunoassay (AutoDelfia; PerkinElmer, Turku, Finland). A random selection of sera from healthy singleton pregnancies from the same study period was used to compare results from the twin pregnancies to the singletons (n = 474).

Table 1 — Baseline characteristics of monochorionic and dichorionic twin pregnancies in this study. Since we did not dispose complete data on ethnicity and IVF these were not included in the table.

	Monochorionic	Dichorionic	*p*-value
n	116	650	
median gestational age (days)	85	83	0.002
median maternal age (years)	34.6	35.1	0.675
median maternal weight (kg)	68	70	0.354
smoking (%)	3.4	8.3	0.069

For each marker the concentrations were expressed in multiples of the gestation-specific normal median (MoMs). Normal medians were obtained by regression of the observed median for each completed week of gestation in unaffected singleton pregnancies against the median days, weighted by the number of controls. The observed MoM value was divided by the expected value for maternal weight, based on the regression of the observed median MoM according to weight group against 1/weight, weighted by the number of controls. Standard deviations were calculated based on the log-transformed weight corrected MoMs. Furthermore, MoMs were corrected for smoking status. Analysis of variance (ANOVA) and additional Bonferroni post-hoc analysis was used to analyze differences in log-transformed weight corrected MoM values of PAPP-A and fβ-hCG for different gestational weeks. Non-parametric Mann–Whitney U tests (two-tailed) were used to analyze differences between MoM values of all markers in monochorionic and dichorionic twin pregnancies. Correlation coefficients were calculated for the associations of $\log_{10}$ MoMs of all markers, after excluding outliers exceeding 3 standard deviations from the median. P-values <0.05 were considered statistically significant.

Results

In *Table 1* the baseline characteristics of the monochorionic and dichorionic twin pregnancies are shown.

A significant difference was seen for gestational age; however, the difference in medians was only 2 days. Since we did not dispose complete data on ethnicity and in vitro fertilization (IVF), we could not compare these.

Table 2 shows the median MoM values and $\log_{10}$ MoM standard deviations of all markers in both monochorionic and dichorionic twin pregnancies as compared to singletons. For none of these markers a significant difference in MoM values between monochorionic and dichorionic twins was found. Therefore, for subsequent analyses data of monochorionic and dichorionic twins were merged.

Table 2 — Median MoM (standard deviation $\log_{10}$ MoM) of PAPP-A, fβ-hCG, ADAM12 and PP13 for monochorionic and dichorionic twin pregnancies, and singletons. MoMs were corrected for maternal weight and smoking status. Mann–Whitney U tests were performed to analyze the difference between monochorionic and dichorionic twin pregnancies.

	Singletons	Monochorionic	Dichorionic	*p*-value
n	69749	116	650	
PAPP-A	1.00 (0.26)	2.03 (0.23)	2.18 (0.25)	0.133
fβ-hCG	1.00 (0.26)	1.87 (0.24)	1.89 (0.23)	0.746
n	1269	51	249	
ADAM12	1.00 (0.15)	1.66 (0.15)	1.64 (0.13)	0.965
PP13	1.00 (0.18)	1.56 (0.17)	1.53 (0.17)	0.272

The median MoMs and $\log_{10}$ MoM standard deviations of all markers for every gestational week are shown in *Table 3*. A trend towards increasing MoM values during the first trimester was seen for PAPPA. ANOVA analyses showed a significant increase of PAPP-A ($p = 0.01$) between gestational weeks. Additional Bonferroni post-hoc analyses showed that this significant difference was between MoMs of weeks 9–11 and 9–12, respectively. There was no significant relationship between gestational age and fβ-hCG, ADAM12 and PP13 MoMs. Moreover, no such trend was observed for singleton pregnancies (data not shown). Correlation coefficients were calculated for all marker combinations in all twin pregnancies. PAPP-A was highly correlated with ADAM12 ($r = 0.573$; $p < 0.0001$) and PP13 ($r = 0.423$; $p < 0.0001$) and to a lesser extent with fβ-hCG ($r = 0.130$; $p = 0.025$). There was also a strong correlation between ADAM12 and PP13 ($r = 0.398$; $p < 0.0001$). Furthermore, fβ-hCG was significantly correlated with ADAM12 ($r = 0.131$; $p = 0.02$) and PP13 ($r = 0.236$; $p < 0.0001$). In two twin pregnancies Down syndrome was present in one of the foetuses and in one other twin pregnancy trisomy 13 was found in one foetus (all three were dizygous

and therefore dichorionic pregnancies). One of the two Down syndrome pregnancies was detected through first-trimester screening. In the first Down syndrome case all MoM values were considerably increased (PAPP-A MoM = 3.46; fβ-hCG MoM = 5.72; ADAM12 MoM = 2.34; PP13 MoM = 2.20) compared to median MoM values of healthy twin pregnancies. In the second Down syndrome case PAPP-A was decreased (MoM = 1.23). ADAM12 and PP13 were at most slightly decreased (MoM = 1.41 and MoM = 1.36, respectively) and fβ-hCG was normal (MoM = 2.07). In the trisomy 13 case all markers (PAPP-A MoM = 1.19; fβ-hCG MoM = 1.30; PP13 MoM = 0.84) were decreased, except for ADAM12 (MoM = 1.58).

Table 3 — Median MoM (standard deviation log_{10} MoM) of PAPP-A, fβ-hCG, ADAM12 and PP13 in all twin pregnancies for every gestational week.

GA (week)	*n*	PAPP-A	fβ-hCG	*n*	ADAM12	PP13
8	5	2.36 (0.67)	1.36 (0.45)	1	0.90 (0.00)	2.38 (0.00)
9	58	1.82 (0.31)	1.82 (0.22)	26	1.39 (0.16)	1.58 (0.24)
10	157	1.92 (0.28)	1.90 (0.22)	47	1.64 (0.15)	1.53 (0.19)
11	161	2.19 (0.23)	1.72 (0.22)	58	1.65 (0.13)	1.54 (0.18)
12	271	2.35 (0.21)	1.91 (0.23)	124	1.63 (0.13)	1.48 (0.15)
13	114	2.08 (0.20)	1.98 (0.25)	44	1.72 (0.11)	1.85 (0.14)
		$p = 0.01$	$p = 0.047$		$p = 0.121$	$p = 0.230$

Discussion

In this study distributions of four first-trimester biochemical markers were established for monochorionic and dichorionic twin pregnancies. The median MoMs of PAPP-A and fβ-hCG were approximately twice as high as compared to singleton pregnancies and were comparable to medians found in previous studies [46, 236, 237, 239, 241, 243, 244]. The median PAPPA MoMs in the current study tended to increase during the first trimester. This was not observed for PAPP-A in singleton pregnancies (data not shown). There was no matching tendency for fβ-hCG, ADAM12 and PP13. As gestational errors cause PAPP-A MoM to increase with gestation but fβ-hCG MoM to decrease, gestational errors are not a likely explanation for this trend. Therefore, a physiological effect in first-trimester twin pregnancies would be more likely to cause the increased MoMs.

During the study period the Dutch Down syndrome screening programme did not officially include screening for twin pregnancies and screening was only performed on specific request. Therefore, the number of twin pregnancies in this study neither represents the incidence of twins in the Dutch population nor the proportion of monochorionic and dichorionic twin pregnancies.

Table 4 summarizes previous studies on differences in screening markers between monochorionic and dichorionic twin pregnancies [236, 237, 239, 241]. Recently Spencer *et al.*, and

Table 4 — Overview of the differences in median MoM values of PAPP-A and fβ-hCG in monochorionic and dichorionic twin pregnancies of four studies.

		n	PAPP-A	fβ-hCG
Goncé *et al.* [236]	Monochorionic	11	2.17	1.67
	Dichorionic	87	1.92	1.54
	p-value		0.07	0.8
Linskens *et al.* [237]	Monochorionic	37	1.59	1.53
	Dichorionic	163	2.4	2.11
	p-value		0.003	0.002
Spencer *et al.* [241]	Monochorionic	190	1.76	1.98
	Dichorionic	1024	2.25	2.04
	p-value		<0.0001	0.93
Current study	Monochorionic	116	2.03	1.87
	Dichorionic	650	2.18	1.89
	p-value		0.133	0.746

Niemimaa et al. [239] and Wøjdemann et al. [242] found a non-significant difference of PAPP-A and fβ-hCG between monochorionic and dichorionic pregnancies, but did not report MoMs or p-values. Therefore, these studies were not included here.

Linskens *et al.* in a smaller study, reported a significantly lower PAPP-A MoM in monochorionic compared to dichorionic twin pregnancies. In our study, no statistically significant difference was found in PAPP-A or fβ-hCG MoMs between monochorionic and dichorionic twin pregnancies, which was also reported by Goncé *et al.*, Wøjdemann *et al.* and Niemimaa *et al.* For ADAM12 and PP13 as well, we did not observe a significant difference in median MoMs between monochorionic and dichorionic twin pregnancies. Since most biochemical screening markers are predominantly produced by the placenta and the placental mass of monochorionic and dichorionic twins are comparable at term [247], and therefore possibly also in the first trimester, differences in MoMs between monochorionic and dichorionic twins should not necessarily be expected. However, if a significant difference in marker concentrations between monochorionic and dichorionic twin pregnancies exists, it would be expected for either all or none of these markers. Nevertheless, the observed discrepancy between studies currently remains unresolved.

Strikingly, median ADAM12 and PP13 MoMs in twin pregnancies were only approximately 1.5 times increased compared to singleton pregnancies. The presence of two foetuses should normally lead to a factor two increase in MoM values, as reported for PAPPA, fβ-hCG and AFP [238, 244, 248]. However, for unconjugated estriol (uE3) a median MoM of 1.68 has been reported in a meta-analysis [249], but this was attributed to simultaneous maternal production of uE3. Both ADAM12 and PP13 have been found to be decreased in singleton

pregnancies complicated by pre-eclampsia (PE) [210, 212, 250]. The low value of these proteins in twin pregnancies may be related to the three-fold increase of PE in multiple gestations and/or to the increased incidence of third trimester foetal growth restriction [251, 252]. On the other hand, the median PAPP-A MoM in our study was normal (data not shown) but was expected to be also slightly decreased if many PE complicated pregnancies had been included [253]. In this study, no information on PE nor birth weight centile was available. In future studies it should be investigated if indeed low ADAM12 and PP13 levels can be explained by a high incidence of PE and/or growth restriction, and if the former can be used to predict the onset of the latter two.

In this study MoM values were not corrected for ethnicity since this is not commonly applied in the Netherlands. As a result, MoM values could be underestimated [254]. However, in less than 5% of all twin pregnancies in this study mothers were reported as non-Caucasians (data not shown). In addition, MoM values were not corrected for IVF. This information was not available until 2008 and was therefore not taken into account. From the available data we derived that approximately 25% of all dichorionic twins and 10% of all monochorionic twins were conceived by IVF. Since PAPP-A MoMs are decreased in IVF pregnancies [255], correction for this parameter, assuming these percentages for the entire study population, would probably lead to an even smaller difference between monochorionic and dichorionic twin pregnancies in this study.

Correlation coefficients of the markers in twin pregnancies were comparable to those of singleton pregnancies [59, 155, 223]. In aneuploid singleton pregnancies, PAPPA, ADAM12 and PP13 MoMs tend to be decreased and the fβ-hCG MoM is mostly increased [37, 38, 223, 245]. These changes should also be expected in aneuploid twin pregnancies. In this study, variation in MoMs of the aneuploid twin pregnancies is large with too few cases to comment on aneuploid MoM distributions.

In conclusion, this study provides insight in the distributions of PAPP-A, fβ-hCG, ADAM12 and PP13 for twin pregnancies. If ADAM12 and PP13 will be adopted as first-trimester screening markers, the presented median MoM values, standard deviations and correlation coefficients for twin pregnancies may contribute to a proper twin risk estimation.

Part III | Chapter 10

Discovery of novel serum markers for Down syndrome screening by integrative data mining

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Abstract

Background To facilitate the experimental search for novel maternal serum biomarkers in prenatal Down syndrome screening, we aimed to create a set of candidate biomarkers using a data mining approach.

Methodology/Principal Findings Because current screening markers are derived from either foetal liver or placental trophoblasts, we reasoned that new biomarkers can primarily be found to be derived from these two tissues. By applying a three-stage filtering strategy on publicly available data from different sources, we identified 49 potential blood-detectable protein biomarkers. Our set contains three biomarkers that are currently widely used in either first or second trimester screening (AFP, PAPP-A and fβ-hCG), as well as ten other proteins that are or have been examined as prenatal serum markers. This supports the effectiveness of our strategy and indicates the set contains other markers potentially applicable for screening.

Conclusions/Significance We anticipate the set will help support further experimental studies for the identification of new Down syndrome screening markers in maternal blood.

Introduction

For over two decades, prenatal screening for Down syndrome (DS) has been available to pregnant women. A screening procedure usually consists of a risk calculation based on maternal serum measurements and other parameters like nuchal translucency and maternal age, after which women with a high predicted risk can opt for invasive testing such as amniocentesis or chorion villus sampling. Initially, the most commonly used method for risk calculation was the second trimester triple test, which combines serum levels for alpha-fetoprotein (AFP), unconjugated estriol (uE3), and the free beta subunit of human chorion gonadotropin (fβ-hCG) with maternal age [16, 32]. In recent years, this test has been largely replaced by the first-trimester combined test, which is based on fβ-hCG and pregnancy-associated plasma protein A (PAPP-A) serum concentrations, ultrasound nuchal translucency (NT) measurements and maternal age [43]. The latter test is the method currently available to pregnant women in the Netherlands.

Despite international experimental effort to improve the DS screening, both the detection rate (DR) and false positive rate (FPR) can still significantly be improved upon. In the Netherlands, the current DS screening has a DR of 75.9% and an FPR of 3.3% [54]. Most research effort in this field is focused on finding new biomarkers for which serum levels can be added to the risk calculation algorithm. In recent years, proteomics methods for large-scale protein quantitation have been employed to facilitate the search for such biomarkers [151, 256-260]. However, the performance of candidate biomarkers obtained by such studies are not always reproducible, and also established DS pregnancy biomarkers are not always successfully confirmed in such studies, likely due to issues related to technical sensitivity and reproducibility.

A recent study by our group used bead-based multiplexed immunoassays to test 90 different analytes in first-trimester maternal serum samples from DS pregnancies and controls [151]. This study identified seven new potential biomarkers that allow for a more accurate first-trimester risk prediction, while confirming the long-known usefulness of PAPP-A. The set of 90 analytes was not pregnancy- or DS- specific but based on a pre-fixed commercially available set. We reasoned that with a set that is more focused on markers relevant for pregnancy or DS, more and also more specific biomarkers can be found. Hence, we set out to develop such a set by analysis and integration of publicly available data.

The amount of information on genes and proteins in databases is increasing rapidly, which allows for a bioinformatics approach that involves automated collecting and combining information from biological databases, known as data mining. Recent studies using data mining for identification of blood based cancer biomarkers showed the successfulness of this approach [261, 262].

Current DS screening protein biomarkers can be traced to originate from two tissues,

namely foetal liver (e.g. AFP) and the placenta (e.g. fβ-hCG, PAPP-A). The non-protein serum biomarker uE3, routinely used in second trimester screening, is produced by the placenta from its precursor dehydro-epiandrosterone sulphate derived from the foetal adrenal glands and liver [263, 264]. Placental markers can be assigned more specifically to the trophoblast cells, which are involved in both the implantation of the embryo into the endometrium as well as the production of hormones required for establishing and sustaining pregnancy. Indeed, abnormal trophoblast differentiation has been observed in placentas of DS pregnancies [81, 265]. As the current screening biomarkers are all derived from the two tissues mentioned (i.e. foetal liver and placental trophoblast cells), we hypothesized that several novel useful biomarkers can primarily be found to be derived from these two tissues. To identify such protein biomarkers we combined data from several publicly available sources.

Methods

Analysis of Tissue-Specific Gene Expression Data

Human tissue-specific gene expression data were analyzed using the Symatlas web-interface (http://symatlas.gnf.org) based on data previously published by Su *et al.* [266, 267]. Symatlas data were considered most useful for this study as it provides the largest publicly accessible data collection on multiple tissues, including both adult and foetal tissues. Tissue data used were both from the Human GeneAtlas GNF1H (gcRMA-normalised) (79 tissues) and the Human GeneAtlas U95A (44 tissues). Using the web-interface, these data sets were queried for the number of genes highly expressed in foetal liver or placenta, according to different stringency levels. Such a stringency level consists of a minimal ratio for the gene expression in a target tissue (in our case placenta or foetal liver) compared to the median expression of that gene across all tissues examined. Using various stringency levels, the number of tissue-specific genes obtained for each stringency level was determined for foetal liver as well as placenta. The resulting data were imported into the statistical programme R (www.R-project.org) [268] and the data distribution was assessed to determine the non-specific underlying trend over lower stringency levels. This revealed that for lower thresholds this trend could be approximated with a power law distribution, where a two-fold increase in the threshold led to a four-fold decrease in the number of genes expressed above that threshold. We refer to this trend as the non-specific underlying trend. For higher stringencies, the number of genes began to decrease at a slower rate, indicating an enrichment for tissue-specific genes over the non-specific trend. Based on this finding, a threshold was set that yielded approximately 10 times more tissue-specific genes than could be estimated based on the non-specific underlying trend. In other words, using the trend for non-specific genes at lower stringency levels, a stringency threshold was chosen that was high enough to consider 90% of the genes highly expressed in either foetal liver or placenta to be specifically derived

from that tissue and not be a statistical artefact. These genes were used in subsequent analysis steps. The same approach was used to enrich foetal liver-specific genes compared to adult liver-specific genes.

Text Mining

As text mining is still a developing field, we wanted to include more than one text-mining tool to restrict the chance of false negatives. For that reason two applications were combined as they use different approaches to search partially different databases, and therefore can be considered complementary. The first of these is Anni (http://www.biosemantics.org/anni/) [269], which provides an ontology- and thesaurus-based interface to Medline and retrieves associations for several classes of biomedical concepts (e.g. genes, drugs and diseases). These concepts are given a concept weight, which indicates their relevance to the applied search term. The second application is Polysearch (http://wishart.biology.ualberta.ca/polysearch) [270], which supports different classes of information retrieval queries against several different types of text, scientific abstract or bioinformatics databases such as PubMed, OMIM, DrugBank, SwissProt, the Human Metabolome Database (HMDB), the Human Protein Reference Database (HPRD), and the Genetic Association Database (GAD). The relevancy scores of the obtained genes or proteins are expressed as Z scores, i.e. as standard deviations above the mean. The two applications were searched for genes associated with the terms “trophoblast”, “cytotrophoblast” and “syncytiotrophoblast”. Significance criteria for Anni were based on a minimally tenfold enrichment over the statistically determined distribution of the concept weight. For PolySearch, a Gaussian distribution was used, based on the software documentation. Gene lists obtained for the three terms were combined and subsequently manually adjusted to resolve ambiguous or redundant gene symbols.

Assessing Applicability for Blood-Based Detection

To determine if putative biomarkers identified by gene expression analyses and/or text mining are potentially blood-detectable, they were cross-checked against two different data resources. Proteins were considered blood-detectable if they had at least one of the Gene Ontology (GO) annotation terms ‘‘extracellular region’’, ‘‘extracellular region part’’, or ‘‘extracellular space’’; or if they were included in the Human Plasma Proteome (HPP) list. GO (http://www.geneontology.org) [271] annotations are partially based on computational predictions whereas the HPP list [272] is based on a combination of experimental methodologies. The latter approach revealed some blood-detectable proteins not predicted by Gene Ontology, but lacked some low-abundance proteins including protein hormones [272]. As with the text mining tools, these approaches were therefore considered complementary and results were combined.

Results

Identification of Tissue-Specific Candidate Genes

The DS screening biomarkers currently implemented in the first-trimester combined test or the second trimester triple test are derived from two tissues, namely foetal liver and placental trophoblasts. Therefore, the first step in our data mining approach consisted of identifying genes specifically expressed in either one of these two tissues (see *Figure 1* for an overview of the various selective steps).

The tissue-related gene expression resource Symatlas was searched for genes expressed in either foetal liver or placenta, at a level that is a (user-definable) multiple of the median expression for that gene across all tissues. By using various threshold levels and statistical analysis, we found that at a gene expression threshold of 30 times the median tissue expression, ten times more genes were identified by the Symatlas query than were expected based on the non-specific trend. This applied to foetal liver as well as placenta. Therefore, we used this criterion to select genes that have a high probability of being specific for these individual tissues. This approach resulted in 158 proteins specific for foetal liver and 229 for placenta, respectively (*Figure 1*).

Applying Additional Relevance Criteria

The second step consisted of further prioritizing our set of genes by ensuring that the genes selected in step 1 are not only highly expressed, but are also sufficiently relevant for the tissues mentioned. In the case of foetal liver-specific genes, we again used Symatlas to ensure that the expression in foetal liver exceeded at least ten times that of adult liver, thus narrowing down the list from 158 to 51 genes (*Figure 1*).

For placenta-specific genes we used two complementary text mining tools (Anni and Polysearch) to select genes related to three trophoblast-related search terms (trophoblast, cytotrophoblast, syncytiotrophoblast). For Anni, genes with a concept weight > 0.0001 (based on yielding ten times more terms than expected for a non-specific distribution) and for Polysearch genes with a Z value > 1.6 for at least one of the terms were selected. This way, 181 genes were found, 50 of which were also found to overlap with the previous selection of 229 placenta-specific genes (*Figure 1*). We applied a different filtering method for the two tissues because Symatlas does not include gene expression data specific to trophoblasts or its two subtypes, whereas text mining was less able to make a distinction between proteins related to foetal or adult liver.

The 51 foetal liver-specific genes and 50 trophoblast-related genes were subsequently analyzed for detectability in blood.

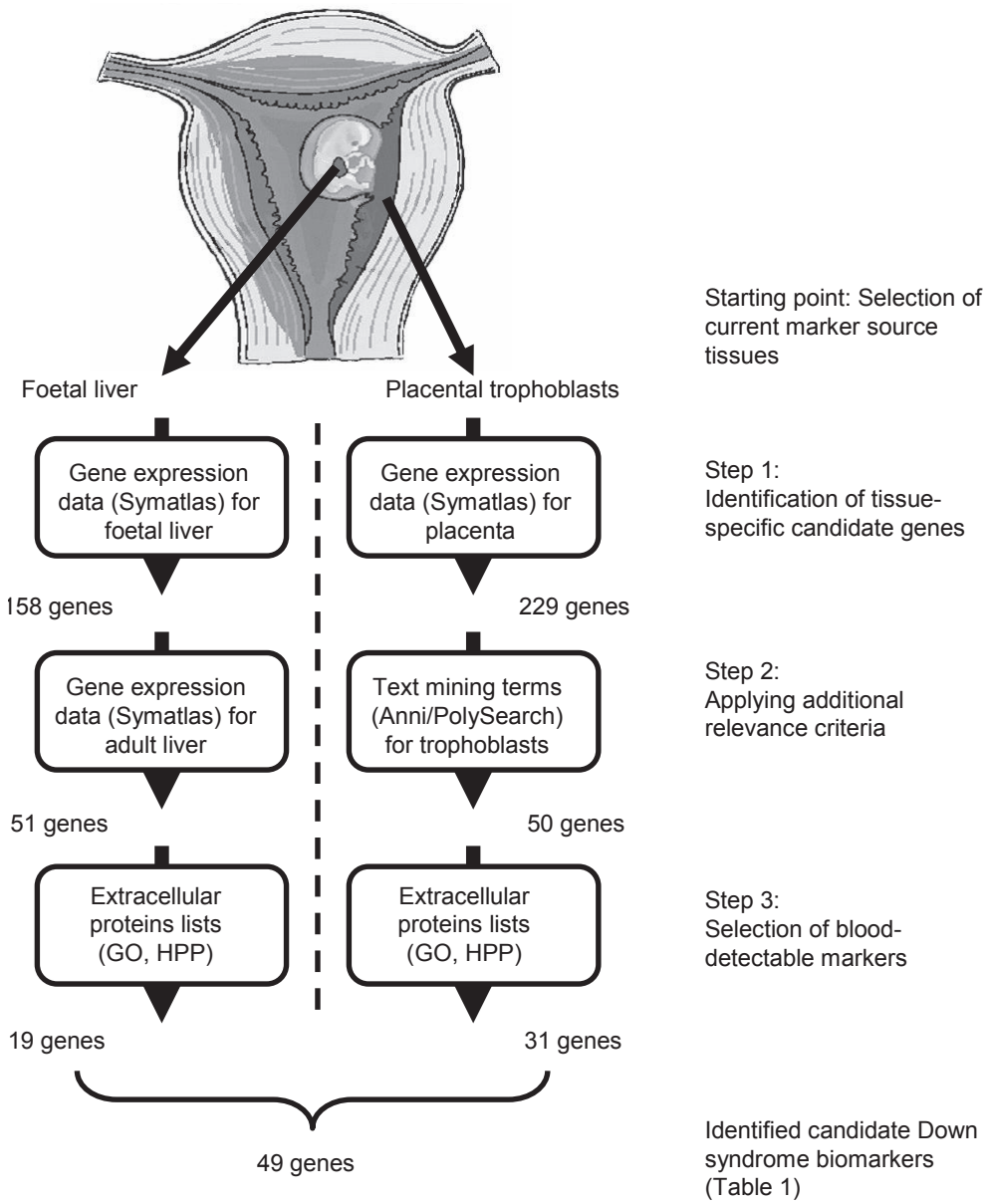

Figure 1 – Schematic representation of the steps employed in our data mining strategy and the number of genes selected after each step.

Selection of Blood-Detectable Markers

For implementation of a biomarker in a routine human screening program, it is essential that it can be detected in serum or plasma. For the markers selected by the previous steps,

we examined which ones had a Gene Ontology annotation as being extracellular, or were part of the experimentally derived Human Plasma Proteome list compiled by Anderson *et al.* [272]. This final selection step resulted in 49 individual blood-detectable markers (*Figure 1, Table 1*). For foetal liver and placenta, these numbers were 19 and 31, respectively, with IGF2 being part of both sets (*Figure 1, Table 1*).

Discussion

The aim of this study was to design a set of new potential blood-detectable biomarkers for prenatal DS screening by computational data mining that is more focused on DS screening than currently available commercial multiplex kits or high-throughput methods for whole proteome analysis. By combining data from different (publicly available) data sources into a three-stage approach (summarized in *Figure 1*), we identified 49 of such protein markers (*Table 1*). Our combined list contains three biomarkers that are currently widely used in either first- or second-trimester DS screening, namely AFP, PAPP-A and fβ-hCG. This demonstrates that the method used is able to identify relevant DS screening biomarkers. In addition, the list contains several other proteins which have been examined for their potential as DS screening biomarkers by several research groups, such as the inhibin chains INHA and INHBA [273-276], the (protein-identical) placental lactogen genes CSH1 and CSH2 [160], placental growth hormone (GH2) [152, 277, 278], placental growth factor (PGF) [279], IGFBP1 [152], or PP13 (LGALS13) [223]. For five of these proteins (INHA, CSH1, CSH2, GH2, PGF), significant differences in concentration exist between DS and euploid pregnancies, and therefore these can be used as a biomarker in DS screening. Additionally, two collagen-related markers, COL1A1 and COL3A1, (as well as IGFBP1) have been described to have different amniotic fluid levels in DS pregnancies [260] and it is conceivable that this also applies to the corresponding maternal serum levels although this remains to be established.

While identifying AFP, PAPP-A as well as fβ-hCG as DS screening biomarkers, our approach failed to identify the second trimester biomarker unconjugated estriol (uE3). However, as uE3 is not a protein biomarker, it is not supported by our strategy based on gene expression and protein data integration. Another biomarker that our approach failed to detect but has been described in the literature is ADAM12. This protein is both highly expressed in placenta and extracellular, but failed the criteria used in the text mining step. It should be noted, however, that most recent studies find this biomarker to be informative only before 10 weeks of gestation, so the applicability of this protein is already limited [155, 280-283]. The finding that two complementary text mining methods did not find sufficient evidence for association of ADAM12 with trophoblasts can either indicate that current literature databases only provide weak evidence for this association, or that both text mining tools were not successful in detecting an existing association. As text mining is a developing field, both options are plausible. A recommendation for future studies of this kind might therefore be to consider including further text mining tools based on additional search algorithms.

Table 1 – Identified candidate Down syndrome (DS) biomarkers.

Symbol	Chrom	Description	Potential
Fetal liver-derived markers			
AFP	4	alpha-fetoprotein	In use
ANGPTL3	1	angiopoietin-like 3	
C5	9	complement component 5	
COL1A1	17	collagen, type I, alpha 1	Indications
COL1A2	7	collagen, type I, alpha 2	
COL2A1	12	collagen, type II, alpha 1	
COL3A1	2	collagen, type III, alpha 1	Indications
COL5A2	2	collagen, type V, alpha 2	
DEFA3	8	defensin, alpha 1	
DLK1	14	delta-like 1 homolog (drosophila)	
ELA2	19	elastase 2, neutrophil	
GPC3	X	glypican 3	
IGF2	11	insulin-like growth factor 2 (somatomedin A)	
PF4	4	platelet factor 4 (chemokine (C-X-C motif) ligand 4)	
PPBP	4	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	
RRM2	2	ribonucleotide reductase M2 polypeptide	
S100A8	1	S100 calcium binding protein A8 (calgranulin A)	
S100A9	1	S100 calcium binding protein A9 (calgranulin B)	
SPTA1	1	spectrin, alpha, erythrocytic 1 (elliptocytosis 2)	
Placental trophoblast-derived markers			
ADM	11	adrenomedullin	
ALPP	2	alkaline phosphatase, placental	
CDH1	16	cadherin 1, type 1, E-cadherin (epithelial)	
CDH11	16	cadherin 11, type 2, OB-cadherin (osteoblast)	
CGA	6	glycoprotein hormones, alpha polypeptide	
CGB5	19	chorionic gonadotropin, beta polypeptide (**fβ-hCG**)	In use
CRH	8	corticotropin releasing hormone	
CSH1	17	chorionic somatomammotropin hormone 1 (placental lactogen)	Biomarker
CSH2	17	chorionic somatomammotropin hormone 2	Biomarker
EBI3	19	Epstein Barr virus induced gene 3	
EGFR	7	epidermal growth factor receptor	
FN1	2	fibronectin 1	
GH1	17	growth hormone 1	
GH2	17	growth hormone 2	Biomarker

IGF2	11	insulin-like growth factor 2 (somatomedin A)	
IGFBP1	7	insulin-like growth factor binding protein 1	Examined
INHA	2	inhibin, alpha	Biomarker
INHBA	7	inhibin, beta A (activin A, activin AB alpha polypeptide)	Examined
INSL4	9	insulin-like 4 (placenta)	
LGALS13	19	lectin, galactoside-binding, soluble, 13 (PP13)	Examined
PAPPA	9	pregnancy-associated plasma protein A, pappalysin 1	In use
PGF	14	placental growth factor	Biomarker
PLAC1	X	placenta-specific 1	
PLAU	10	plasminogen activator, urokinase	
PRL	6	prolactin	
PSG5	19	pregnancy specific beta-1-glycoprotein 5	
SERPINB2	18	serpin peptidase inhibitor, clade B, member 2	
SERPINE1	7	serpin peptidase inhibitor, clade E, member 1 (PAI1)	
SPP1	4	secreted phosphoprotein 1 (osteopontin)	
TGFB1	19	transforming growth factor, beta 1	
TIMP3	22	TIMP metallopeptidase inhibitor 3	

Potential for DS screening is indicated as follows: In use, currently widely used in first or second trimester DS screening (in bold); Biomarker, studies showed overall significant concentrations; Examined, examined as biomarker but not significant or inconclusive overall results; Indications, found in high-throughput study but awaiting further study. References on the corresponding literature are given in the Discussion.

Among the 49 proteins in *Table 1*, several over-representations of biological processes can be observed. Among the foetal liver-derived genes the five collagen genes are most apparent, but there are also a number of proteins related to innate immunity such as C5, PF4, PPBP, S100A8, and S100A9. These immunological proteins can be ascribed to the central role the foetal liver has in foetal haematopoiesis. For the placental trophoblast-derived proteins the majority act as hormones or growth factors, and in addition four proteins (PLAU, SERPINB2, SERPINE1, and TIMP3) are involved in tissue remodelling. Both these processes are associated with the role of placental trophoblasts in the production of hormones required for establishing and sustaining pregnancy as well as the implantation of the embryo into the endometrium. Given that most of the identified markers are associated with a small number of biological processes, it becomes likely that these pathways might also harbour other potential DS screening markers that do not meet the criteria used in our approach or for which insufficient data are available.

As DS is caused by a (partial) trisomy of chromosome 21, it seems counterintuitive that none of the markers in *Table 1* are located on chromosome 21. Although it might be expected that genes on this chromosome are expressed at an approximately 1.5-fold higher level

compared to other genes and could therefore act as suitable biomarkers, this assumption does not fully hold in comparative studies [284-286]. Furthermore, proteomic studies, including our own, found no increased presence of chromosome 21 proteins among the differentially expressed proteins [151, 256-260]. Moreover, although partial trisomy 21 is sufficient for DS, efforts to associate DS with a smaller chromosomal region have excluded the possibility of a single region being responsible for all aspects of the phenotype [287-290]. Additionally, several characteristics of a DS phenotype are also found for other types of aneuploidy, indicating that the higher expression of genes located on chromosome 21 is only linked indirectly to DS phenotype and mainly acts through dysregulation of genes on other chromosomes. This can also explain why current DS screening biomarkers are not located on chromosome 21 and the pregnancy screening biomarkers in use are also predictive for other chromosomal aberrations such as Edwards syndrome (trisomy 18) and Patau syndrome (trisomy 13). This actually creates the possibility that some of the markers mentioned in *Table 1* are not only applicable to DS screening, but also to pregnancies with other types of foetal aneuploidy.

By means of integrative data mining we have derived a set of candidate Down syndrome screening biomarkers. As the first two filtering steps are both based on a minimally ten-fold enrichment or induction over the corresponding background, we expect the number of false positives, i.e. not relevant markers, to be low. This is corroborated by the presence of eight proteins in our set that are in use or can be used as biomarker for DS screening and five other proteins for which this has been studied. However, before biomarkers can be tested in a large-scale cohort study, additional serum analysis experiments will be necessary to validate which of these candidate biomarkers have differential levels in DS versus normal pregnancies. Furthermore, we cannot predict beforehand at what gestational age biomarkers are most discriminatory between normal and DS pregnancies, and as a result, whether they can be integrated in late first or early second trimester screening. If this proves not to be the case, the usability of the new biomarkers in a routine, large-scale population screening programme as applied in the Netherlands will be rather low. These further experimental validations of the new DS screening biomarkers identified by our data mining approach will evidently be the subject of future follow-up studies.

Part III | Chapter 11

Bead-based multiplexed immunoassays to identify new biomarkers in maternal serum to improve first-trimester Down syndrome screening

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Abstract

Objectives To identify new discriminative biomarkers for Down syndrome (DS) pregnancies using a bead-based multiplexed immunoassay, and to use the newly identified biomarkers to construct a prediction model for non-invasive DS screening.

Methods Maternal serum samples of 14 DS pregnancies and 15 matched controls were analyzed with a bead-based multiplexed immunoassay containing immunoassays for 90 different analytes. Potential biomarkers were selected on the basis of concentration fold ratios between DS and control samples. For these markers and the current screening markers (pregnancy-associated plasma protein A [PAPP-A], free beta subunit of human chorion gonadotropin [fβ-hCG] and nuchal translucency) prediction values were obtained and used to calculate detection rates (DR) at a 5% false positive rate.

Results Seven potential biomarkers of which the fold ratio exceeded 1.3 or −1.3 were selected for further analysis. All 14 DS cases in this study were detected using the combination of all currently used and newly identified markers. The modelled DR for all markers extrapolated to the general pregnant population was 82.5%, compared to a modelled DR of 56.2% for the current screening markers.

Conclusion This study demonstrates the possibility of improving the performance of the current first-trimester DS screening by addition of new biomarkers, which were identified using bead-based multiplexed immunoassays.

Introduction

For many years, screening for Down syndrome (DS) during pregnancy has been carried out using a risk calculation algorithm based on biochemical and biometric parameters. Currently, the most popular algorithm is the one used in the so-called first-trimester combined test, performed between 8 and 13 weeks of gestational age. This test is composed of the concentrations of pregnancy-associated plasma protein A (PAPP-A) and the free beta subunit of human chorion gonadotropin (fβ-hCG) in maternal serum, the nuchal translucency measurement (NT) and maternal age. Women with a high risk test result can opt for invasive prenatal diagnosis such as amniocentesis or chorion villus sampling (CVS). With the first-trimester combined test, 75–85% of all DS cases are detected at a false positive rate (FPR) of 5% [53, 59, 60].

Much effort has been put into improving the first-trimester combined test by strict quality assurance, e.g. the quality assurance of the NT measurement [176, 183]. Still, the detection rate (DR) and FPR allow for further improvement. Currently, a significant number of pregnant women are wrongfully reassured or wrongfully referred for invasive prenatal diagnosis, where the latter is associated with at least a 0.1% chance of an iatrogenic abortion [26, 27].

There are several ongoing studies on new non-invasive techniques for DS screening, using, e.g. foetal DNA and foetal cells in maternal blood [61-63]. Another and possibly cheaper and more applicable method to improve first-trimester DS screening is by means of multiple marker analysis in serum. The new discriminative markers to be used for this approach can be identified using innovative proteomics approaches.

Proteomics is the large-scale study of proteins with regard to their structure, functional characterization and quantification. Proteomics methods allow a large number of proteins to be studied simultaneously in order to obtain accurate and comprehensive data and to correlate expression-level changes of proteins. Among others, proteomics is used to detect biomarkers for a specific disease or syndrome. As such, proteomics techniques may also be suitable to identify new biomarkers for DS screening since certain proteins can be found in maternal blood through placental diffusion. New potential DS screening markers have been found and tested individually, e.g. ADAM12, PlGF and leptin [149, 154, 161]. However, although initial results seemed promising, newly identified single biomarkers have not significantly improved the performance of the screening up to now. Recently, two-dimensional (2-D) gel electrophoresis and tandem mass spectrometry (MS–MS) have been used to detect several potential biomarkers in amniotic fluid and maternal blood [257, 258, 260, 291], clearly demonstrating the potential of applying proteomics techniques in searching for new biomarkers.

We hypothesized that an accurate set of (new) biomarkers could contribute to an improvement of the detection of DS. With novel techniques such as bead-based multiplexed immunoassays it is possible to analyze many potential markers simultaneously and process a large set of sera relatively rapidly. This method is cost effective and easy to implement in existing screening facilities, and as such deserves to be explored for new possibilities in DS

screening.
The Dutch National Institute for Public Health and the Environment (RIVM), as a reference laboratory for DS screening, possesses an extensive collection of sera of pregnant women carrying a DS foetus. To identify potential biomarkers, samples from this serum base were used in a bead-based multiplexed immunoassay approach. Subsequently, a prediction model was constructed to determine the level of improvement when these markers were included in non-invasive DS screening.

Methods

Sample Selection
From the RIVM serum bank of 2006, sera of 15 pregnancies with DS and 15 unaffected pregnancies were selected. A first-trimester combined screening test had been performed for all these pregnant women. The control samples were matched to the cases by gestational age (exact day), maternal weight (± 5 kg) and maternal age (± 1 year). Blood samples were drawn between 84 and 90 days of gestational age. One sample was excluded from analysis because it appeared (post-selection) to originate from a pregnancy with a vanishing twin.

Serum Analysis
The two currently used serum markers of the first-trimester combined test (PAPP-A, fβ-hCG) were already measured in the selected samples at the RIVM using an automated dissociation-enhanced lanthanide fluorescent immunoassay (AutoDelfia; PerkinElmer, Turku, Finland). For this study, the serum samples were analysed by bead-based multiplexed immunoassay at the biomarker testing laboratory Rules Based Medicine (RBM; Austin, Texas, USA). RBM uses multi-analyte profiling (MAP) to measure multiple proteins in small sample volumes (± 100 μL) for multiple species and sample types and is certified according to the Clinical Laboratory Improvement Amendments (CLIA). For this analysis, the HumanMap Version 1.6, which is a pre-existing non-pregnancy-specific immunoassay, was used, containing immunoassays for 90 different analytes. The samples were blinded and randomized prior to analysis.

Data Analysis
Of the 90 analytes, 17 were excluded, since in more than 50% of them the concentration of the specific analyte was below the detection range. For other analytes, values flagged as being below the detection range were replaced for further calculations by an estimated concentration of 80% of the lowest measurable concentration for that analyte. For the remaining 73 analytes, the fold ratio between the geometric average concentrations of the cases and the controls was calculated. The fold ratio was defined as the higher value divided by the lower value, with a plus or minus sign indicating an increase or decrease in DS pregnancy serum levels.

Table 1 – Overview of the fold ratios of the concentrations of the Down syndrome cases compared to matched controls for all measurable analytes of the RBM multiplexed assay. At the bottom of the table are the fold ratios of the three current screening markers analysed at the RIVM using a dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA). The analytes marked bold were selected for further analysis (fold ratio >1.3 or –1.3).

Antigen	Fold Ratio	Antigen	Fold Ratio
Alpha-1 Antitrypsin	1.020	Interleukin-16	1.036
Adiponectin	-1.090	Interleukin-18	1.261
Alpha-2 Macroglobulin	1.124	Interleukin-1 beta	1.167
Alpha-Fetoprotein (AFP)	**-1.398**	Interleukin-1 ra	1.018
Apolipoprotein A1	-1.127	Interleukin-7	1.006
Apolipoprotein CIII	-1.006	Interleukin-8	1.222
Apolipoprotein H	1.002	**Insulin**	**-1.391**
Beta-2 Microglobulin	1.086	Leptin	1.131
Brain-Derived Neurotrophic Factor	-1.095	**Lipoprotein (a)**	**-2.004**
Complement 3	1.028	MCP-1	1.113
Cancer Antigen 125	1.070	MDC	1.159
Cancer Antigen 19-9	1.016	MIP-1alpha	-1.055
CD40	-1.048	MIP-1beta	1.075
CD40 Ligand	-1.030	MMP-3	-1.110
Carcinoembryonic Antigen	-1.005	Myeloperoxidase	-1.111
Creatine Kinase-MB	1.029	Myoglobin	-1.205
C Reactive Protein	-1.018	PAI-1	-1.072
Epidermal Growth Factor (EGF)	**-1.719**	Prostatic Acid Phosphatase	-1.021
ENA-78	1.211	**PAPP-A**	**-2.688**
EN-RAGE	**-1.371**	RANTES	-1.208
Eotaxin	**1.369**	Serum Amyloid P	1.013
Fatty Acid Binding Protein	1.099	Stem Cell Factor	1.055
Factor VII	-1.065	SGOT	1.068
Ferritin	1.258	Sex Hormone Binding Globulin	-1.176
FGF basic	-1.178	Thyroxine Binding Globulin	-1.089
Fibrinogen	-1.040	Tissue Factor	1.115
G-CSF	1.095	TIMP-1	-1.018
Growth Hormone	1.182	Tumour Necrosis Factor RII	1.044
Glutathione S-Transferase	-1.016	Tumour Necrosis Factor-alpha	1.181
Haptoglobin	**1.525**	Thrombopoietin	1.053
ICAM-1	1.050	Thyroid Stimulating Hormone (TSH)	-1.256
Immunoglobulin A	-1.130	VCAM-1	1.008
Immunoglobulin E	1.148	VEGF	1.037
IGF-1	-1.061	von Willebrand Factor	1.038
Immunoglobulin M	-1.081		
Interleukin-10	-1.149	**fβ-hCG**	**1.637**
Interleukin-12 p70	-1.080	**PAPP-A**	**-1.936**
Interleukin-13	-1.111	**MoM NT**	**1.427**
Interleukin-15	-1.099		

Analytes of which the fold ratio exceeded 1.3 or −1.3 were selected for further analysis. In this subsequent analysis with the selected potential biomarkers, the current screening markers PAPP-A, fβ-hCG and NT were also taken into account. In the current first-trimester combined test maternal age is also a parameter. In this study, the maternal age was not taken into account as a parameter in the model, since both sample groups were matched for maternal age.

For the selected analytes, the log-transformed concentrations were adjusted for the average of the control sample concentrations for that analyte. Adjusted concentrations of all selected analytes were combined and used to construct a least-squares fit linear regression model for predicting the DS cases, using the statistical programme 'R' (www.R-project.org) [268]. Thus, prediction values based on different marker combinations were calculated for each case and control. By plotting these prediction values, the discriminatory power between cases and controls in the study cohort was evaluated. Assuming that the prediction values would be normally distributed in both DS and control pregnancies, the DR at a 5% FPR could be estimated for different marker combinations. By doing so, the data of this relatively small cohort were extrapolated to the general pregnant population.

Results

Mean maternal age in the 14 DS cases was 36.3 ± 1.6 years compared to 36.0 ± 1.5 in the 15 controls. Mean maternal weight was 86 ± 1 kg for DS and 87 ± 1 kg for controls. All women were Caucasian and non-smokers. Nine out of 14 DS cases were classified as high risk for DS based on current DS screening, while none of the controls was classified as high risk.

For 73 analytes, the fold ratio of their concentration in the DS cases compared to the controls was calculated. The fold ratios of the three current screening markers were also included (*Table 1*).

From this large number of analytes tested, seven potential biomarkers in which the fold ratio exceeded 1.3 or −1.3 were selected for further analysis: alpha-fetoprotein (AFP), epidermal growth factor (EGF), extracellular rage binding protein (EN-RAGE), eotaxin, haptoglobin (HP), insulin (INS) and lipoprotein A (LPA). PAPP-A, which was already included in the current routine screening test, was also one of the analytes in the RBM assay. Interestingly, both PAPP-A fold ratios were highly comparable (*Table 1*). Since the objective of this study was to improve the current DS screening, PAPP-A levels measured with the AutoDelfia (see also the Methods section) were used for further analysis. For each of the selected markers, the concentrations adjusted for the geometric average concentration of the controls were plotted (*Figure 1*).

None of the new markers was in itself fully discriminatory between the DS cases and the controls. To extend the possibilities of the marker set, a comparison was made between the

predictive value for DS of the current screening markers, the seven potential biomarkers and a combination of these (*Figure 2*). The difference in medians of the DS and control samples was larger when 10 markers were used compared to the 3 current screening markers only. More importantly, when 10 markers were used there was no overlap of the upper limit of the controls and the lower limit of the DS cases, which provided in this data set a full distinction between cases and controls.

Under the assumption of normal distributions in both DS and control pregnancies, a modelled DR at a 5% FPR was calculated for different marker combinations (*Table 2*). In line with previous analyses, this analysis also did not reveal a single marker which in itself significantly improved the screening performance beyond that of the current test.

Interestingly, all 14 cases in this study were detected through the combination of all 10 markers compared to 9 out of 14 cases using the current screening. Using the set of 10 markers, the modelled DR at a 5% FPR was 82.5%, which is a considerable improvement over the DR of the three current screening markers in this model (56.2%). Even when the NT measurement, which is a strong marker in current DS screening, was left out of the model, the DR was considerably better (78.9%).

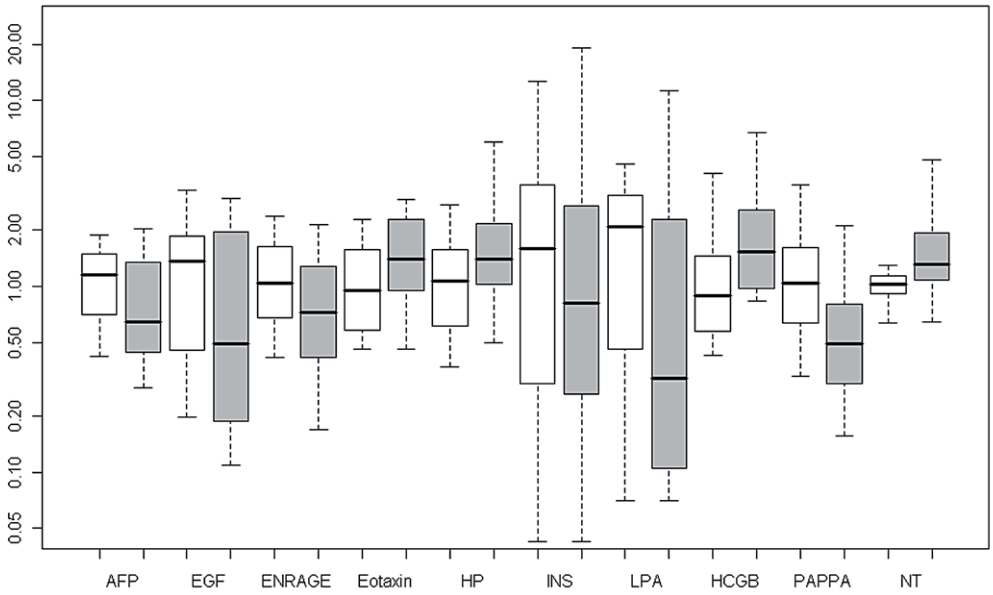

Figure 1 – Boxplots showing the adjusted marker concentrations of all seven newly identified biomarkers and the three current screening markers by plotting the median, quartiles and minimum/maximum values. In white: control samples (average ratio is 1.0), in grey: Down syndrome samples.

Discussion

The main goal of this study was to identify new serum markers potentially applicable for DS screening. A case–control study was conducted using sera from DS pregnancies and matched controls. Using bead-based multiplexed immunoassays, seven new potential biochemical markers were identified on the basis of the concentration fold ratio between cases and controls. Possibly, more analytes with smaller ratios could also have been useful biomarkers, especially if the spread of those analytes was small in cases and controls. In this study we chose to select analytes based on high fold ratios, as in our view these markers are analytically probably more robust and easier to validate.

The combination of 10 markers provided a full distinction (100%) for the small set of DS cases and controls in this study. After extrapolating the data to the general pregnant population, the DR at a 5% FPR was 82.5% compared to 56.2% for the current screening.

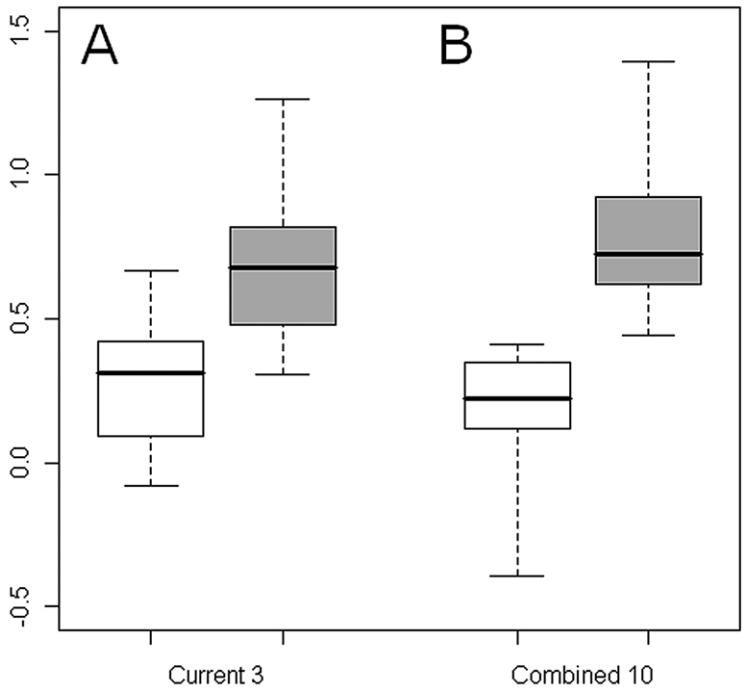

Figure 2 – Boxplots showing the distinction between Down syndrome cases (grey) and the controls (white) by plotting the median, quartiles and minimum/maximum values. (A) Difference between cases and controls when the three current screening markers (PAPP-A, fß-hCG and NT) are used, (B) Difference between cases and controls when the current three screening markers are combined with the seven newly identified potential biomarkers (AFP, EGF, EN-RAGE, Eotaxin, HP, INS and LPA). Values along the vertical axis indicate prediction scores expressed as arbitrary units.

Of the 14 DS sera analyzed, 5 were from women who received a low-risk test result for carrying a child with Down syndrome. Interestingly, the proposed set of 10 markers would have been able to identify these cases also. This result demonstrates that an extension of the biochemical marker panel tested is, in principle, capable of reducing the number of false negative test results, although sample numbers tested were relatively small.

None of the identified and subsequently selected proteins is linked to genes located on chromosome 21. However, some of the markers are known to be highly expressed in the placenta or foetal liver. The decrease of AFP in DS pregnancies has been described previously, mainly in the second trimester of pregnancy [16, 292]. HP has also been associated with DS in earlier studies [293, 294]. Foetuses with DS have abnormalities in their lipid metabolism [295], which could explain the decreased levels of LPA. INS and EGF, both decreased, are involved in placental development, which is thought to be impaired in DS pregnancies [76, 81]. Insulin is the primary determinant of insulin-like growth factor binding protein-1 (IGFBP-1) expression. IGFBP-1 was found to be significantly decreased in amniotic fluid of DS pregnancies [260], which suggests a functional connection to corresponding changes in insulin levels in these pregnancies. EGF is a growth factor that regulates cytotrophoblast differentiation and invasion during early pregnancy and has an anti-apoptotic effect [296]. EN-RAGE and eotaxin are both immunologic factors and no relationship with DS has been described before. However, several publications have reported abnormalities of the immune system in people with DS [297, 298].

Table 2 – Modelled detection rate (DR) for the general population based on the 14 analysed Down syndrome cases and 15 control samples, at 5% false positive rate (FPR). Note that maternal age was not taken into account in the model.

	modelled DR (%) at 5% FPR
PAPPA, fβ-hCG, NT (current screening)	56.2
Current + AFP	58.9
Current + EGF	62.6
Current + ENRAGE	58.7
Current + Eotaxin	61.1
Current + HP	61.8
Current + INS	59.7
Current + LPA	61.0
All new markers	38.7
Only serum markers	78.9
Current + new markers	82.5

None of the individual selected analytes showed a major fold change in DS as compared to controls. This is in line with most previous studies using other markers or techniques that also

did not reveal high fold changes [257, 258, 260]. Plausible explanations for this phenomenon might be that biomarkers with major fold changes are still awaiting identification or, alternatively, that fold changes are inherently not high in maternal blood.

One of the aims of this study was to demonstrate the potential of bead-based multiplexed immunoassays for DS screening. The costs of these assays containing 10 markers are approximately the same as the current AutoDelfia method in which only two markers are tested. Additionally, with bead-based multiplexed immunoassays, many markers can be analyzed simultaneously in a high-throughput analysis setting. Thus, this technique can be a cost-effective screening method.

For DS screening, the NT is considered to be a good, albeit difficult-to-measure, parameter demanding rigorous monitoring of the quality of this measurement [176, 183]. In this study it was shown that with a high-quality set of biomarkers it might even be possible to exclude the NT measurement or put it to use more effectively, e.g. in a contingent screening setting. Although the combination of our newly identified set of biomarkers with the three current markers already leads to a considerable improvement of the detection rate, one has to keep in mind that these biomarkers were derived from a pre-fixed immunoassay that is not directly related to pregnancy and/or DS. With an assay more targeted to pregnancy- and DS-related markers, it is likely that more markers will be identified, presumably further improving the test performance. On the other hand, test performance is always better when a screening test is applied to the same cases from which the markers were derived: application of our markers of a different cohort of cases is needed to establish the diagnostic accuracy of the immunoassay we propose.

Overall, this study demonstrates the possibilities of improving the performance of current first-trimester DS screening using a multiple marker approach. Future efforts will focus on a larger set of sera, a wider range of gestational age and a marker selection more dedicated to pregnancy and DS. Further research also includes biomarker screening for other foetal aneuploidies, e.g. trisomy 13 and trisomy 18.

Part III | Chapter 12

Proteomics and Down syndrome screening: mixed blessings from a validation study

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In revision

Abstract

Objective In a previous discovery study we identified seven potential screening markers for Down syndrome (DS). Here, we report on an extended study to validate the discriminative potential of these markers and to search for additional ones.

Methods Concentrations of 45 analytes were measured using bead-based multiplexed immunoassays in maternal serum from 34 DS pregnancies and 33 matched controls. Prediction values were obtained for current screening markers (PAPP-A, fβ-hCG and NT), seven markers identified before and newly discovered markers based on concentration fold ratios between DS and controls. Models were fitted based on data of the discovery study or the current study and also tested on both datasets.

Results Significantly higher fold ratios were only found for EGF (-1.96; $p = 0.006$) and CA19-9 (-2.29; $p = 0.004$). In the prediction model for the current dataset EGF, EN-RAGE and CA19-9 together improved the detection rate of DS with 16.2% (at a fixed 5% false positive rate) when added to the currently used screening markers.

Conclusions Validation of previously identified biomarkers only confirmed EGF for further consideration as a DS screening marker. This underlines the importance of validating biomarkers, in this study limiting the range of plausible biomarkers to only a few suitable ones.

Introduction

Current Down syndrome (DS) screening is mostly performed by first-trimester assessment of pregnancy-associated plasma protein A (PAPP-A) and the free beta subunit of human chorion gonadotropin (fβ-hCG) in maternal serum combined with a sonographic nuchal translucency (NT) measurement and maternal age. This results in the detection of 75-85% of all DS pregnancies at a 5% false positive rate (FPR) [53, 54, 59].

In recent years, proteomics techniques have been used to search for additional biomarkers which may improve sensitivity and specificity of first-trimester DS screening when added to the serum assay. Until now a few studies on DS marker identification using proteomics have been performed, including one of our own group [151, 257, 258, 260, 291]. In these studies 8 to 56 DS cases were included and potential screening markers were identified in either amniotic fluid, maternal plasma or maternal serum. However, in most of these studies only the proteins' fold-changes between DS and control samples were presented, and no results concerning the predictive power of (combinations of) these proteins in a screening algorithm were presented [257, 258, 260]. Moreover, in the one study in which a prediction model was constructed, the method used (SELDI-TOF) does not allow for more accurate protein identification, so the underlying potential DS screening markers remained unidentified [291]. Our own group recently performed a discovery study in which seven new potential biomarkers for DS were identified. With these new markers, in addition to the current DS screening markers, a full distinction could be made between the 14 DS and 15 control pregnancies [151].

Typically, biomarker discovery studies have limited generalizability and sample size but are perfectly satisfactory to establish a proof of principle. However, these studies are often liable to all sorts of bias, which makes it necessary to validate the results in larger studies [299, 300]. A study to validate the potential DS screening markers found in the previously described studies has not been published so far. Therefore, the aim of our current study was to analyze the seven proteins identified in our discovery study in a larger set of DS and control sera, to evaluate their potential as screening markers more extensively and to validate the prediction model in a larger set of DS cases. Moreover, the multiplexed immunoassays were used to identify additional potential biomarkers.

Methods

Sera of 34 DS pregnancies and 34 unaffected pregnancies from the serum bank of the Dutch National Institute for Public Health and the Environment (RIVM) were retrieved from -80 °C storage. A first-trimester combined screening test (serum analysis of PAPP-A and fβ-hCG, and a NT measurement) had been performed between 2004 and 2007 for all these pregnancies. The control samples were matched to the cases by gestational age (exact day), maternal weight (± 5 kg) and maternal age (± 1 year). All blood samples were drawn in the

12th week of pregnancy (between 84 and 90 days of gestational age).
Two currently used serum markers in the first-trimester combined test (PAPP-A, fβ-hCG) were already measured in the selected samples at the RIVM using an automated dissociation-enhanced lanthanide fluorescent immunoassay (AutoDelfia; PerkinElmer, Turku, Finland). The seven potential screening markers identified in our earlier study were alpha-fetoprotein (AFP), epidermal growth factor (EGF), extracellular rage binding protein (EN-RAGE), eotaxin, haptoglobin (HP), insulin (INS) and lipoprotein A (LPA). Several commercially available bead-based multiplexed panels were used to analyze these proteins: Widescreen Human Cancer Panel 1+2 and Widescreen Human CVD Panel 5+6, Novagen, Merck KGaA, Darmstadt, Germany; Human Serum Adipokine Panel B and Human Cytokine/Chemokine Panel (Eotaxin) and Beadlyte Human Cancer Biomarker Panel Kit (IGF-II), Millipore Corporation, Billerica, MA, USA. Most panels also contained other analytes besides the seven analytes of interest, these were therefore also measured. This way, a total of 45 analytes were examined in all 68 serum samples. Standard analysis protocols were followed and all samples were analyzed singularly.
Of the 45 analytes, four were excluded, because in more than 50% of the samples the concentration of the specific analyte was below the detection range. For other analytes, values flagged as being below the detection range were replaced for further calculations by an estimated concentration of 90% of the lowest measurable concentration for that analyte in any given sample. One sample from the control group had to be excluded since the amount of serum appeared too little for proper analysis. Thirteen of the selected samples (seven in the DS group and six in the control group) were also used in the discovery study by Koster *et al.* [151], and were re-analyzed in the current study to verify earlier results. To explore the robustness of these bead-based analyses, Pearson correlation coefficients were calculated to compare the log-transformed serum concentrations of the seven analytes of interest with their serum concentrations measured in the discovery study.
For all 41 analytes the fold ratio between the geometric average concentrations of the cases and the controls was calculated. The fold ratio was defined as the higher value divided by the lower value, with a plus or minus sign indicating an increase or decrease in DS pregnancy serum levels. Student's t-tests were performed to calculate whether fold ratios were statistically significant.
Further validation of the seven markers identified in our previous study as part of a DS prediction model was performed by calculating prediction scores models similarly as described in our previous study [151]. For these models, current screening markers PAPP-A, fβ-hCG and NT were included, in combination with one or more additional markers. The seven previously identified markers were all selected for this further analysis irrespective of whether their fold ratio was statistically significant. Additionally, of the remaining analytes of the current study, those with a significant difference between DS and controls were

selected.

For the selected analytes, the log transformed concentrations were adjusted for the average of the control sample concentrations for that specific analyte. Adjusted concentrations of all selected analytes were combined and used to construct a least squares fit linear regression model for predicting the DS cases, using the statistical programme 'R' (www.R-project.org) [268]. Thus, prediction values based on different marker combinations were calculated for each case and control; higher values represent a higher risk of a DS pregnancy. Rather than calculating a clinical risk, we chose to calculate a score (in arbitrary units), because maternal and gestational age were matched. By plotting these prediction values the discriminatory power between cases and controls in the study cohort was evaluated. Assuming that the prediction values would be normally distributed in both DS and control pregnancies, the DR at a 5% FPR could be calculated for different marker combinations. Prediction models were fitted based on the data of the discovery study or based on the current study and were subsequently tested on both datasets. This way four different calculations were performed: a model fitted on the discovery dataset and tested on the discovery dataset (equivalent to the previous publication [151]), a model fitted on the discovery dataset and tested on the current dataset and vice versa, and finally, a model both fitted and tested on the current dataset.

In the current first-trimester combined test maternal age is also a parameter. In this study maternal age was not taken into account as a parameter in the model, since both sample groups were matched for maternal age.

Results

Mean maternal age in the 34 DS samples was 37 ± 3 years compared to 36 ± 3 years in the 33 control samples. Mean maternal weights were 73 ± 15 kg and 71 ± 12 kg for DS and control samples respectively. Mean gestational age was 88 ± 2 days in both groups. In the DS group 2 (6%) women were smokers compared to 5 (15%) in the control group. For each woman a first-trimester combined screening test was performed resulting in a risk calculation of having a child with DS. In the DS group 20 (58.8%) women received a high risk test result compared to 3 (9.1%) women in the control group considering a cut-off risk of 1 in 200.

To check the reproducibility of the analytes measured in the current study as well as in our previous study, Pearson correlation coefficients were calculated for the seven markers identified before. For all but one analytes a good correlation between serum levels was found; AFP r=0.762, EGF r=0.948, EN-RAGE r=0.802, HP r=0.740, INS r=0.932, LPA r=0.847. However, correlation was poor for Eotaxin (r=0.214).

Fold ratios between DS and controls were significant for PAPP-A (-1.88; p<0.001), fβ-hCG (2.22; p<0.001) and NT (1.50; p<0.001). Of the seven markers previously identified a high

fold ratio was found only for EGF and LPA in the current study. Moreover, only EGF (-1.96; p=0.006) was statistically different between groups. Furthermore, among the additional analytes measured in this study a significant high fold ratio was found for CA19-9 (-2.29; p=0.004) (*Table 1*).

Next, a comparison was made between the predictive values for DS of the current screening markers (PAPP-A, fβ-hCG and NT) alone and in combination with one or more newly discovered potential screening markers. Under the assumption of normal distributions in both DS and control pregnancies a modelled DR at a 5% FPR was calculated for different marker combinations (*Table 2*). Of the seven markers identified in our previous study EGF and EN-RAGE again showed an improved DR of approximately 3-4% when added to the current three screening markers (PAPP-A, fβ-hCG and NT) in the validation dataset. Interestingly, as shown in *Table 1*, EN-RAGE by itself had no significant high fold-change (-1.19; p=0.385), but in combination with other markers did improve DR. When combining both EGF and EN-RAGE with the three current screening markers, DR increased at least 4.9% and at most 14.1% depending on the fitted model used.

CA19-9 was found to be significantly decreased in the DS samples and its predictive value was therefore evaluated. Since CA19-9 was not one of the analytes in the discovery study only one model could be used for this potential marker. Adding CA19-9 to the model containing PAPP-A, fβ-hCG, NT, EGF and EN-RAGE increased the DR from 71.4% to 80.2%. When the NT measurement was left out of the prediction model the DR was still 73.2% compared to 57.6% for the model using only the current biochemical screening markers PAPP-A and fβ-hCG. The distinction between DS and controls for the model with the three current screening markers and the two most predictive models (current screening markers + EGF + EN-RAGE (+ CA19-9)) is shown in *Figure 1*. From this boxplot it is clear that when the new potential markers are added to the prediction model, the overlap between the upper limit of the controls and the lower limit of the DS cases becomes smaller, which indicates a larger distinction between DS and controls.

Discussion

This was the first validation study in which biomarkers for DS detected with proteomics techniques in a population have been tested in a new population.

Since the multiplexed immunoassays used in this study were different from those in the discovery study, correlation coefficients were calculated to compare serum concentrations of the analytes of interest between the two individual studies. All but one of those correlations appeared good, implying that experimental procedures and resulting data were highly comparable. The poor correlation of Eotaxin could be due to the use of different antibodies in the two assays, known to affect experimental outcome because of differences in affinity.

Table 1 – Overview of the fold ratios and p-values for all measured analytes in DS compared to unaffected pregnancies. The potential screening markers with a statistically significant difference are displayed in bold.

Marker	Fold Ratio	*p*-value	Marker	Fold Ratio	*p*-value
PAPP-A	**-1.88**	**<0.001**	*Fibrinogen*	1.05	0.867
fβ-hCG	**2.22**	**<0.001**	*HB-EGF*	1.07	0.541
NT	**1.50**	**<0.001**	*HGF*	-1.12	0.195
AFP	-1.05	0.741	*IGF-II*	1.10	0.285
EGF	**-1.96**	**0.006**	*IL-6*	-1.39	0.351
EN-RAGE	-1.19	0.385	*IL-8*	-1.22	0.356
Eotaxin	-1.08	0.730	*Leptin*	1.23	0.285
Haptoglobin	1.01	0.887	*MCP-1*	1.09	0.423
Insulin	1.22	0.352	*NGF*	-1.22	0.624
Lipoprotein (a)	-1.52	0.181	*PAI-1*	1.01	0.944
Adiponectin	1.01	0.913	*PDGF-BB*	1.17	0.123
A2M	-1.04	0.557	*PlGF*	-1.28	0.113
CA 125	-1.13	0.485	*Prolactin*	1.14	0.394
CA 15-3	-1.10	0.527	*SAP*	-1.03	0.731
CA 19-9	**-2.29**	**0.004**	*Tenascin C*	-1.02	0.928
CEA	-1.37	0.168	*TGF-alpha*	1.31	0.427
CRP	1.29	0.310	*TNF-alpha*	-1.10	0.323
Cystatin C	1.00	0.981	*VCAM-1*	1.06	0.619
EGFR	-1.01	0.833	*VEGF*	1.06	0.436
Epiregulin	1.00	0.993	*vWF*	-1.06	0.612
Fetuin-A	-1.06	0.323			

As expected, fold ratios between DS cases and controls were high for the current first-trimester DS screening markers PAPP-A, fβ-hCG and NT. Of the seven analytes previously identified a high fold ratio could only be confirmed for EGF. In the current study the fold ratio was -1.96 compared to -1.72 in the discovery study. For the other analytes, the high fold ratios in the discovery study could not be reproduced. AFP, which is a screening marker for DS in the second trimester of pregnancy, but was also reported to be slightly decreased in first-trimester DS pregnancies [301, 302], could not be confirmed as a screening marker in this study. Moreover, some analytes found to be upregulated in DS cases in the discovery study now showed a downregulation (i.e. Eotaxin, 1.37 compared to -1.08) or the other way around (i.e. Insulin, -1.39 compared to 1.22).

A validation study concerning biomarker discovery for DS screening has never been published, but the lack of reproducibility between initial biomarker discovery and subsequent validation studies has also been reported in other biomarker fields, and may be due to the relatively low number of samples, especially in the discovery studies [300, 303]. In our discovery study 14 DS cases and 15 controls were analyzed, compared to 34 of each group in the current validation study. In the discovery study markers were selected based only on a high fold ratio. However, since none of the high fold changes in the discovery study were significant, some of the findings in that study may have been by chance. This underlines the importance of validating biomarkers, a procedure that in this study limits the range of plausible biomarkers to only a few suitable ones.

Table 2 – Modelled detection rates (DR) at a given 5% false positive rate (FPR) for several marker combinations. Models were fitted based on the data of our earlier study (discovery fit) or based on the current study (validation fit) and tested on both datasets. CA19-9 was not measured in the first study; therefore a model based and tested only on the current data had to be used to calculate DR for marker combinations including CA19-9. DRs displayed in bold indicate an improvement compared to the current screening model.

	DR at a 5% FPR			
markers in the model	discovery fit + discovery data	discovery fit + validation data	validation fit + discovery data	validation fit + validation data
current screening (PAPP-A, fβ-hCG, NT)	56.2	65.2	39.7	64.0
PAPP-A+ fβ-hCG	38.9	57.1	32.7	57.6
current+AFP	**58.9**	64.1	**40.6**	64.0
current+EGF	**62.6**	**67.1**	**51.7**	**68.0**
current+EN-RAGE	**58.7**	**68.4**	**47.7**	**68.1**
current+Eotaxin	**61.1**	55.8	**40.7**	63.8
current+Haptoglobin	**61.8**	62.3	36.1	**64.2**
current+Insulin	**59.7**	65.2	37.4	63.7
current+LPA	**61.0**	65.0	**51.6**	63.4
10 markers (current + 7 new)	**82.5**	42.4	**59.2**	**71.5**
current+EGF+EN-RAGE	**62.1**	**70.1**	**53.8**	**71.4**
current+CA19-9	-	-	-	**73.1**
current+EGF+CA19-9	-	-	-	**75.0**
current+EN-RAGE+CA19-9	-	-	-	**79.1**
current+EGF+EN-RAGE+ CA19-9	-	-	-	**80.2**
EGF+EN-RAGE+CA19-9	-	-	-	36.1
PAPP-A+fβ-hCG+EGF+EN-RAGE+CA19-9	-	-	-	**73.2**

Since we included 13 samples from the discovery study, the validation study cohort was not completely independent. However, a separate data analysis of the independent samples provided similar results as presented here (data not shown). Therefore, we decided to include the entire cohort to strengthen the statistical power of our study.

The prediction model containing 10 markers (current + seven new) based on the discovery data performed considerably worse in the current study (DR = 59.2%) compared to the discovery study (DR = 82.5%). This is presumably due to a prediction model that was likely overfitted on data from the discovery study, since the selected markers in de validation study showed less distinctive fold ratios and predictive values. To prevent overfitting, predictive values of all analytes were also calculated based on the current data.

As expected because of its significant fold ratio, addition of EGF alone already provided a considerable improvement of the DR in all fitted models. EGF is a growth factor produced by the placenta which promotes differentiation and prevents apoptosis in trophoblasts [99, 296]. EGF has been shown to increase syncytial hormone secretion of hCG and is known to be present in high levels in the maternal circulation [100]. Since DS is associated with defective placental development, probably underlying the altered serum concentrations of the current DS screening markers [76, 77], the potential of EGF as a screening marker is conceivable.

Adding EN-RAGE to the current screening model had a similar effect on the DR although its fold ratio did not differ significantly between DS cases and controls. LPA, which was a strong predictor in the discovery dataset and did also show an increased, although not significant fold ratio in the current dataset, showed no improvement of the DR. The strongest predictive model using the smallest number of markers was obtained by adding EGF and EN-RAGE to the current screening markers. This led to an improvement of 5-14% depending on the fitted model.

Besides the seven markers identified earlier, other analytes were also measured in this study. One of those analytes was placental growth factor (PlGF) which has been shown to be decreased in DS pregnancies in previous studies [149, 150]. In our study however PlGF was only slightly, but not significantly, decreased in DS cases and was therefore not used in the prediction model.

An analyte that did show a significant fold change was CA19-9. CA19-9 is a carbohydrate antigen mostly used as a serum marker for malignancies, but it is also present in serum and amniotic fluid of pregnant women [304, 305]. However, a study by Noci *et al.* reported no significant difference in amniotic fluid concentrations of CA19-9 between second trimester DS pregnancies and controls [306], but this might be indicative of the difference in body fluid analyzed (serum vs. amniotic fluid). In our discovery study CA19-9 was not distinctive between DS pregnancies and controls, but in this validation study CA19-9 provided a considerable improvement in DR when added to the previously described model. However, for this potential marker extended validation will, especially given our results presented here, also be necessary.

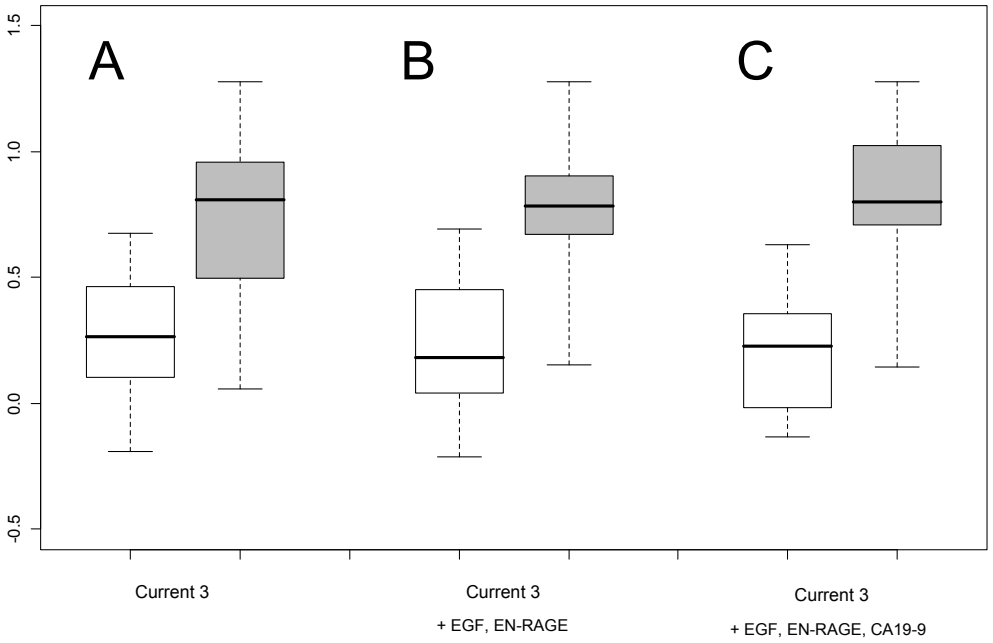

Figure 1 – Boxplots showing the distinction between Down syndrome cases (grey) and controls (white) by plotting the median, quartiles and minimum/maximum prediction values. Distinctions were based on the model fitted on the validation data. (A) Difference between cases and controls when the three current markers (PAPP-A, fβ-hCG and NT) are used. (B) Difference between cases and controls when the three current screening markers are combined with EGF and EN-RAGE. (C) Difference between cases and controls when the current three screening markers are combined with EGF, EN-RAGE and CA19-9. Values along the vertical axis indicate prediction scores expressed as arbitrary units (see methods section).

Despite random sampling of the cohort in this study the DR based on the current screening parameters was relatively low. Usually the DR of the Dutch screening programme is higher [54]. Adding three new markers to the current screening model led to an extra detection of Down syndrome of 16.2% in this population. Therefore, we expect also the DR of the entire Dutch DS screening programme to increase significantly when these markers would be added, but clearly, large scale validation experiments need to be performed to provide evidence for this assumption.

In conclusion, the results of this study were on one hand rather disappointing, since only two of the seven markers from the first study appeared to improve the detection rate of first-trimester DS screening in this subsequent validation study, of which only EGF turned out to be significantly different between DS cases and controls. Apparently, the other results had been chance findings. On the other hand, the finding that two markers again improved

the detection rate of the DS screening in an independent study is highly encouraging. But in summary, data on DS screening using proteomics techniques are scarce and mainly involving few DS cases. Results have, therefore, to be interpreted with caution. That also holds for the new potential marker CA19-9 as found in this study.

Part III | Chapter 13

The quantitative performance of Antibody array technology in a prenatal screening setting

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This thesis

Abstract

Antibody microarrays (Ab-array) represent a new, innovative proteomics platform for high-throughput protein expression profiling in body fluids. Because they allow for multiplexed measurements in small sample volumes, Ab-arrays are an interesting alternative to ELISA if sets of markers are to be analyzed simultaneously. However, to allow implementation of Ab-arrays in clinical or population screening tests, it is of vital importance to establish that this method is both sensitive and quantitative. In this study, we developed and optimized an Ab-array with antibodies against PAPP-A and fβ-hCG, two serum biomarkers currently analyzed by conventional biochemical techniques in prenatal Down syndrome screening. Serum samples derived from pregnant women, representing the dynamic range of these two markers, were analyzed on our designed Ab-arrays, and the concentrations measured were validated to values obtained using the, in prenatal screening routinely applied, AutoDelfia system. Two different array hybridization conditions were tested, i.e. direct and indirect labelling, of which the indirect method displayed a sensitive and quantitative performance and a low intra- and inter-assay variation. Taken together, these findings indicate that Ab-arrays are a promising alternative for ELISA in population screening programs, allowing analysis of multiple biomarkers simultaneously in small volumes of serum.

Introduction

Screening for Down syndrome in the first trimester of pregnancy is based on the maternal serum parameters pregnancy-associated plasma protein A (PAPP-A) and the free beta subunit of human chorion gonadotropin (fβ-hCG) combined with an ultrasonographic nuchal translucency measurement (NT) of the foetus and maternal age. By doing so, 75-85% of all Down syndrome cases are detected at an approximately 5% false positive rate (FPR) [54, 59, 60]. To improve these rates, most research in the field focuses on the discovery of new biochemical markers that can be added to the current screening test. One way of identifying and analyzing these markers is by means of proteomics techniques [151, 257, 258].

The current Down syndrome screening test is based on ELISA methods. ELISA is the golden standard for quantitative protein measurement in serum and can be performed rapidly for a large number of samples. However, ELISA is less suitable to analyze larger sets of markers simultaneously in a high-throughput manner. For this, Antibody microarrays (Ab-arrays) could provide a useful alternative for population screening purposes. Ab-arrays are a high-throughput platform for protein expression profiling in small sample volumes (~10 µL suffices). Small amounts of capture antibodies for the selected targets are immobilized or spotted on a very small area (25-150 µm) on coated glass slides. The high density of the capture antibodies in the spots that is obtained enables high sensitivity with low sample consumption [307]. The method allows for the detection of many markers in parallel, with high sensitivity in small sample volumes. Currently, a wide range of immobilization and detection technologies for arrays is available [308, 309] and the number of purchasable antibodies is increasing. Due to these developments, the potential to generate multiple-marker tests based on Ab-array technology becomes feasible. Multiple marker tests are envisioned to be essential for population screening settings to detect prenatal conditions as well as several diseases (e.g. cancer, heart diseases), since single marker tests usually do not provide for high sensitivity and specificity. Furthermore, Ab-arrays are interesting in the screening field since they allow for large-scale use and only require finger-prick amounts of blood.

Good quantitative performance of a screening test is an important prerequisite for potential future use. Recently, a number of studies on the use of Ab-arrays have been published [308, 310, 311], however, only a limited number of quantitative validation studies have been performed to compare Ab-arrays with ELISA. For our study, we designed an Ab-array consisting of current Down syndrome screening markers (PAPP-A and fβ-hCG) and analyzed its quantitative performance with two different Ab-array methods: *i)* the direct method, where samples are directly labelled with fluorescent dyes; and *ii)* the indirect or sandwich method, which requires two antibodies for the same antigen. One antibody then serves as the capture antibody and is spotted on the surface of the array, while the second (detection) antibody is biotinylated and detected by labelled streptavidin. Results from validation experiments with

sets of serum samples, derived from pregnant women representing the occurring range of the two current biochemical screening markers, will be shown.

Methods

Serum samples and standards for comparative analysis

A set of 16 serum samples from the serum bank of the Dutch National Institute for Public Health and the Environment (RIVM), were retrieved from -80°C storage. A first-trimester screening test for Down syndrome (serum analysis of PAPP-A and fβ-hCG, and a NT measurement) had been performed on these sera in 2008. Samples were selected based on their serum concentrations of PAPP-A and fβ-hCG, measured with an automated dissociation-enhanced lanthanide fluorescent immunoassay (AutoDelfia; PerkinElmer, Turku, Finland). Serum concentrations ranged from the 1% lowest and 1% highest concentration of PAPP-A and fβ-hCG in the screening population. This resulted in serum concentrations ranging from 178-8219 mU/L for PAPP-A and from 9.5-193.4 ng/mL (mU/mL) for fβ-hCG.

For the Ab-array calibration experiments, standards for both PAPP-A and fβ-hCG were obtained from the routinely used AutoDelfia kits. These standards were calibrated against the WHO International Reference Preparation.

Antibody Array design

Monoclonal and polyclonal capture and detection antibodies were purchased: anti-human PAPP-A 10E1 (Hytest, Turku, Finland), anti-human hCG Beta 7 (Acris Antibodies GmbH, Herford, Germany), anti-Cy3/Cy5 (Sigma-Aldrich, St. Louis, MO, USA), anti-human IgG (H+L) (Invitrogen, Breda, the Netherlands), biotinylated anti-human PAPP-A (R&D Systems, Minneapolis, MN, USA) and biotinylated anti-human hCG 28A4 (Hytest). Capture antibodies and BSA were diluted in 2x Protein Array Buffer (Whatman, Kent, UK) to a concentration of 0.1-2.0 mg/ml. The arrays were fabricated using a Piezoarray Non-contact Microarraying System (PerkinElmer, Wellesley, MA, USA), which deposits about 330 pL/drop. The capture antibodies were arrayed by spotting two drops at each position; the first drop was allowed to dry before the second drop was dispensed. The antibodies were spotted in two different concentrations on 16-array nitrocellulose FAST-slides (Whatman). Each array can comprise up to 400 spots and 16 arrays can be spotted on one glass slide. Four replicates of each antibody were arrayed in the same row to ensure adequate statistics. The spotted nitrocellulose-coated slides were incubated overnight at room temperature in a desiccator cabinet (Nalgene, Rochester, NY, USA) and were, subsequently, stored under the same conditions.

Antibody array production

Optimization

For both the direct and indirect method, commercial human serum (Cambrex Corporation, East Rutherford, NJ, USA) was used to optimize spot, label and hybridization conditions. Blocking of surface substrates was optimized using two blocking buffers under different temperature and time conditions; Protein Array Blocking Buffer (Whatman) and Blocker BLOTTO Blocking Buffer (Thermo Scientific, Wilmington, DE, USA). Optimally spotted antibody concentrations for each capture antibody were determined using four different concentrations. Furthermore, two mono-reactive dyes (Cy3 and Cy5), seven labelled serum concentrations, eight hybridization buffers and two wash buffers were tested. For the indirect method, additional conditions regarding the biotinylated detection antibody and the labelled streptavidin concentration were optimized.

Serum labelling

The protein concentrations of the serum samples were determined using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Cy5 labelling of serum was performed according to the manufacturer's protocol (GE Healthcare, Piscataway, NJ, USA). In short, serum samples were diluted in labelling buffer to a concentration of 1 mg/mL protein extract and labelled with Cy5 mono-reactive dye for 30 minutes at room temperature, mixing the solution every 10 minutes. The excess of unreacted dye was removed by passing each solution through SigmaSpin Post-Reaction Clean-up Columns (Sigma-Aldrich). Labelling efficiency was determined by calculation of the Dye-to-Protein Molar ratio (D/P ratio) using a NanoDrop ND-1000 (Thermo Scientific). All D/P ratios were above 2.

Direct method

Slides were mounted in the FAST-frame set-up (Whatman), thus generating 16 independent wells. Based on optimized conditions, the wells were blocked overnight at room temperature with 100 µL Protein Array Blocking Buffer. Subsequently, slides were washed three times for 5 minutes with 90 µL Protein Array Wash Buffer. Next, 90 µL of the labelled serum samples, diluted (250-500 times) in Sigma Washing Buffer (PBS, pH 7.4, 0.05% Tween), were added and the slide was incubated for 30 minutes at room temperature. Finally, the slides were rinsed three times for 5 minutes with Sigma Washing Buffer and for 2 minutes with deionized water. After drying the slides by vacuum, slides were scanned.

Indirect (sandwich) method

Slides were also mounted in the FAST-frame set-up (Whatman). After performing a blocking and washing step, similarly to the direct method, 90 µL of the diluted (10 times) serum sample and the pooled diluted (2 and 10 times respectively) PAPP-A and fβ-hCG standards

was added, and the slide was incubated for 1 hour at room temperature. The slides were washed three times for 5 minutes with Sigma Washing Buffer and incubated with biotinylated PAPP-A and fβ-hCG detection antibodies (diluted 100 and 250 times respectively) for 1 hour at room temperature. Subsequently, the slides were again washed three times for 5 minutes with Sigma Washing Buffer and incubated with Streptavidin-Dylight649 (diluted 500 times; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 minutes at room temperature. Finally, the slides were rinsed three times for 5 minutes with Sigma Washing Buffer and for 2 minutes with deionized water. After drying the slides by vacuum, slides were scanned.

Scanning and data analysis

All slides were scanned with a Confocal Microarray Scanner (PerkinElmer) at a resolution of 10 µm. ScanArray Express software V4.0 was used to quantify the intensity of each spot using the adaptive circle method. Median intensity values, corrected for the corresponding local median background, were extracted for each spot. These data were imported into Microsoft Excel for further analysis. Then, median intensity values of the four replicate spots were calculated. To assess the assay signal quality, signal-to-background levels

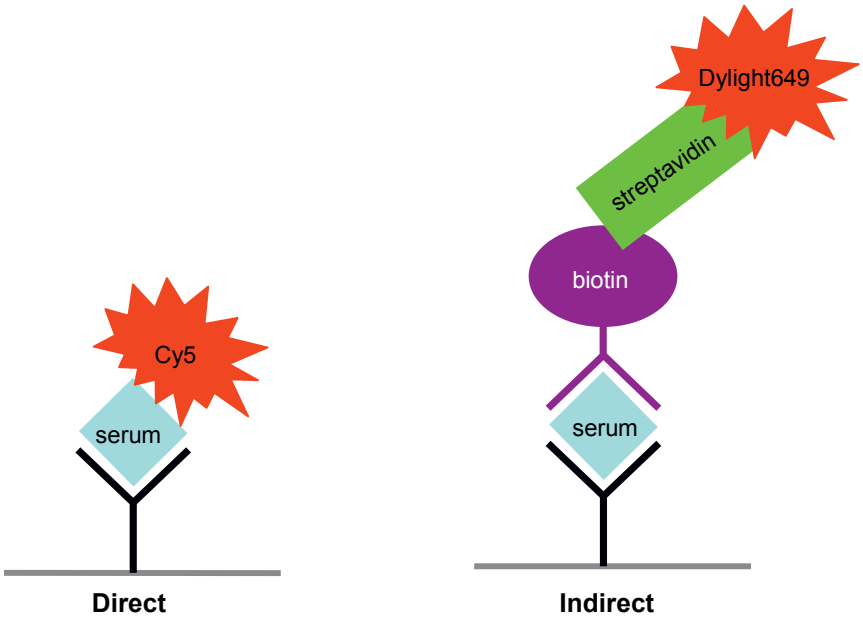


Figure 1 – Schematic representation of direct and indirect Antibody array methods. (A) In the direct method all serum proteins are labelled with fluorescent Dye and incubated on Antibody arrays containing spotted capture antibodies. (B) In the indirect method the serum proteins are captured by antibodies and detected by a cocktail of biotin labelled antibodies in combination with streptavidin labelled fluorescent Dye.

were calculated. For the indirect method, the reproducibility between arrays, slides and batches was calculated using Pearson's correlation and coefficients of variation (CV) (Prism 4, GraphPad software Inc, San Diego, CA, USA). Serum concentrations were calculated from signal intensities using a calibration curve fitted to signal intensities obtained with PAPP-A and fβ-hCG protein standards. Parameter fitting was performed with the statistical programme "R" (www.R-project.org) [268], based on the standard curve model Y(x) = A + (Bx^d /(C+x)), which is a generalization of the Michaelis–Menten model.

Results

Optimization and design of the Antibody array

Before quantitative performance of the Ab-array technology could be accurately assessed, we tested and optimized a variety of general array spotting and labelling methods and materials.

Antibody array spotting

In a series of experiments, different spotting buffers and solutions were tested. Whatman Arraying Buffer as spotting buffer provided the most consistent spot morphology, lowest

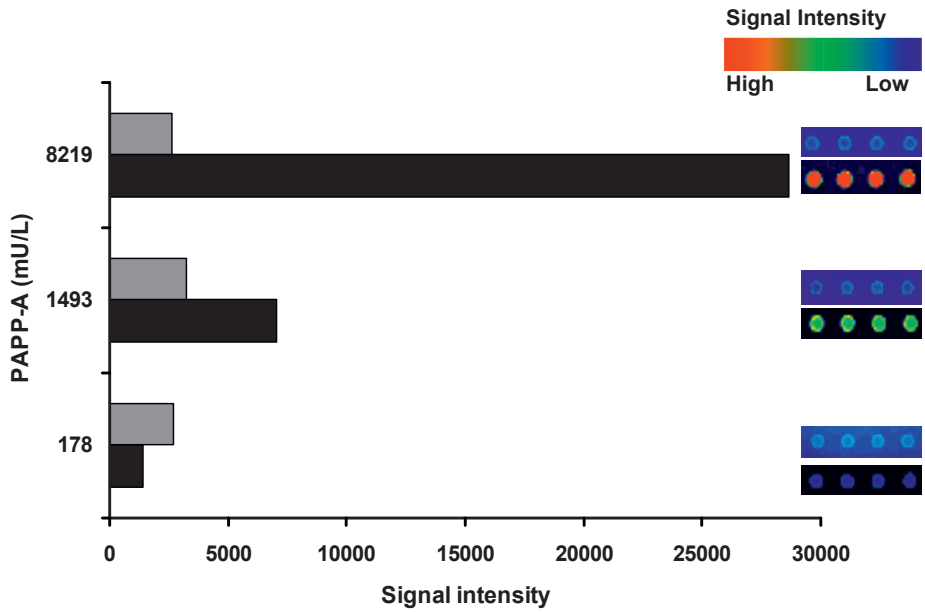


Figure 2 – Quantitative performance of the two Antibody array platforms tested. Antibody array signal intensities of the direct (black bars) and indirect (grey bars) platform are shown for three different PAPP-A serum concentrations.

background, and highest signal-to-background ratios (data not shown). Furthermore, spotting conditions were optimized resulting in humidity below 40%, overnight drying, and storage in a desiccator cabinet. For the comparative and validation experiments described hereafter, these optimized spotting conditions were routinely applied.

Optimization of the direct and indirect labelling method

To obtain functional Ab-array assays for the current Down syndrome screening markers PAPP-A and fβ-hCG, a range of specific optimization experiments were performed. Anti-Cy3/Cy5 and anti-human IgG were used as positive controls and BSA spots as negative controls (aimed at detecting possible non-specific binding). Both the direct and indirect Ab-array platform were optimized (*Figure 1*). Optimized conditions were defined as the best spot signal to background ratio. Overnight (16 hr) blocking at room temperature in Protein Array Blocking Buffer, and incubation and washing steps using Sigma Washing Buffer yielded high signals in combination with low background for both methods (data not shown).

For binding of the capture-antibody to the target (i.e. glass slide), the most optimal spotted antibody concentration was determined. For this, we spotted commercially available antibodies against the two current screening markers PAPP-A and fβ-hCG on each array in different concentrations. BSA was spotted as negative control and anti-Cy3/Cy5 antibody and anti-human IgG were spotted as positive controls, all also with increasing concentrations. Each antibody concentration was spotted in fourfold. The antibody concentration at which the signal appeared saturated was chosen as the optimal concentration for quantitative performance of the arrays. Optimal spotted capture-antibody concentration was, for all cases, 1 mg/ml for anti-PAPP-A, 0.5 mg/mL for anti-fβ-hCG and anti-IgG (H+L) and 0.1 mg/mL for anti-Cy3/Cy5. To determine the optimal detection-antibody concentration, we tested several detection-antibody dilutions (50–2000 times) for consistent performance over the dynamic range of the PAPP-A and fβ-hCG serum concentrations. For the detection-antibodies, the optimal dilution factors were 100 for PAPP-A and 250 for fβ-hCG. For the Streptavidin-Dylight649 optimal dilution factor, we compared several dilutions based on the manufacturer's recommendation. A 500-fold dilution was chosen for further experiments.

Serum concentrations

Optimal serum concentrations were evaluated for both the direct and indirect Ab-array method. For the direct method, a serum concentration diluted 250 times provided the best results (determined here as the lowest CV). For the indirect method, a 10-fold dilution of serum was optimal. Both PAPP-A and fβ-hCG showed good results at this concentration, based on a low CV, a high dynamic range in the detection signal, and the possibility to use one serum dilution for both markers. The latter criterion is important for combining these assays in a multiplex format.

Quantitative performance of the direct and indirect Antibody-array technique

As a first step to test quantitative performance of the direct and indirect array methods over the whole range of PAPP-A and fβ-hCG concentrations, routinely detected in sera from pregnant women, sera with PAPP-A and fβ-hCG concentrations from the lower 1% CI to the upper 1% CI values were selected from the serum bank. Ab-arrays were spotted and optimized conditions were applied for both array methods as described earlier. As shown in *Figure 2*, the indirect labelling method showed good quantitative performance in that signal intensity linearly increased with known, increasing protein concentrations. The direct method showed presence of the proteins but failed to reflect quantitative levels for both proteins that correlated with Cy5 incorporation (data not shown). Consequently, the indirect array method was used for further experiments.

Reproducibility of the indirect Antibody array method

To evaluate assay reproducibility, serum samples with low, intermediate and high levels of PAPP-A or fβ-hCG were selected. The samples were hybridized on 16 arrays on different slides obtained from different spot batches and labelled with Cy5 afterwards. The variation between arrays, expressed as coefficients of variation (CV), was calculated for arrays spotted within one slide and for arrays spotted on different slides (*Table 1*). All but one array showed a CV below 15%, a criterion for FDA approved tests [312]. Accurate performance over the dynamic ranges of both proteins was proved by a high correlation coefficient between arrays and slides (> 0.998; *Figure 3)*. Within 3 different spot batches correlation coefficients were at least 0.9740 for both proteins.

Table 1 – Coefficients of variation (CV), expressed as medians (min CV-max CV), of the indirect Antibody array method between arrays (intra-assay) and between slides (inter-assay).

	Intra-assay variation	Inter-assay variation
PAPP-A	5.7 (1.9-7.0)	8.1 (4.2-11.9)
fβ-hCG	7.0 (1.5-19.6)	7.2 (6.4-12.5)

Assessment of the dynamic range of the indirect Antibody array

The risk calculation for Down syndrome screening requires precise marker concentrations and, therefore, the applicability of the designed Ab-array depends on the quantitative performance over the entire dynamic range of PAPP-A and fβ-hCG. We assessed the quantitative performance of the indirect Ab-array using two different approaches. First, seven arrays were incubated with AutoDelfia standards reaching a dynamic range from 0-10.000 mU/L for PAPP-A and 0-200 ng/mL for fβ-hCG. Both standard curves showed linear detection of the PAPP-A and fβ-hCG concentrations, covering the expected ranges in maternal serum (*Figure 4*). Next, the performance of the Ab-array was validated on the

selected maternal serum samples with a dynamic range from the 1st to the 99th percentile of the population range for PAPP-A and fβ-hCG. Serum concentrations derived from the current AutoDelfia assay and from our Ab-array showed high correlations for both proteins (r = 0.971 for PAPP-A, r = 0.973 for fβ-hCG; *Figure 5*).

Serum analysis on a multiplex array

To evaluate the potential of multiplex analyses of PAPP-A and fβ-hCG, the indirect Ab-array was tested using a combination of the two markers within one array. A cocktail of detection antibodies showed negligible cross-reaction (data not shown), but the combined assay provided, for the entire dynamic range, similar results compared to separate assays. Thus, combination of the current markers within one indirect Ab-array assay reached adequate quantitative performance (*Figure 5*).

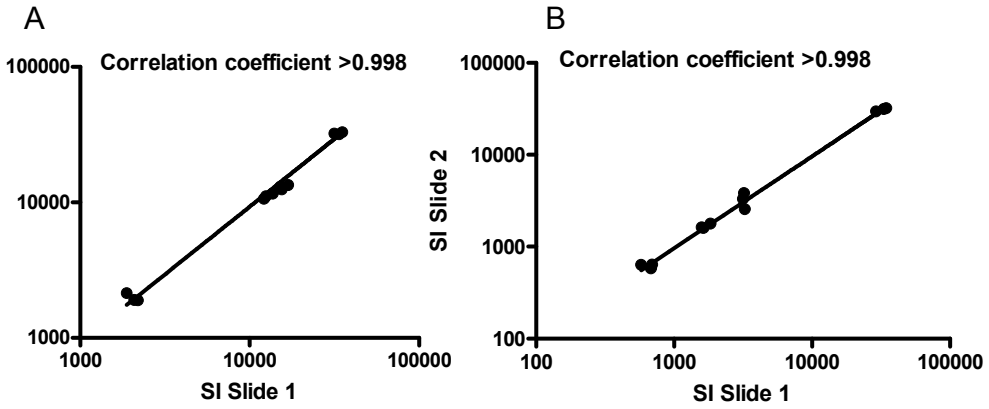


Figure 3 – Array reproducibility of the indirect Antibody array method. Correlations of the signal intensities of PAPP-A (A) and fβ-hCG (B) between different slides are displayed. Each dot represents the median signal intensity of one array.

Discussion

Antibody microarrays (Ab-arrays) can be useful for the large-scale analysis of multiple markers in parallel in small sample volumes. The number of studies describing the use of Ab-arrays is increasing fast, and results from these studies have already shown different potential applications for Ab-arrays, such as cancer detection in serum or other body fluids [309, 313]. Ab-arrays can very well be put to use in population screening settings, in which often several biomarkers are analyzed. An example of such a population screening setting is the prenatal screening for Down syndrome in the first trimester of pregnancy. This screening is currently widely applied; however, sensitivity and specificity can be improved upon by the addition of biochemical markers to the current screening test. But obviously, when more

markers are to be added to the screening test, standard ELISA methods, which can only be used for single marker analysis, are no longer sufficient. Here, we successfully explored the potential of Antibody array technology for prenatal Down syndrome screening, since this technology allows extending the number of biomarkers to be analyzed relatively easily.
Given that the risk calculation for Down syndrome screening requires precise marker concentrations, the applicability of an Ab-array highly depends on its quantitative performance. So far, the quantitative performance of Ab-arrays has not been studied extensively [308, 310, 311].

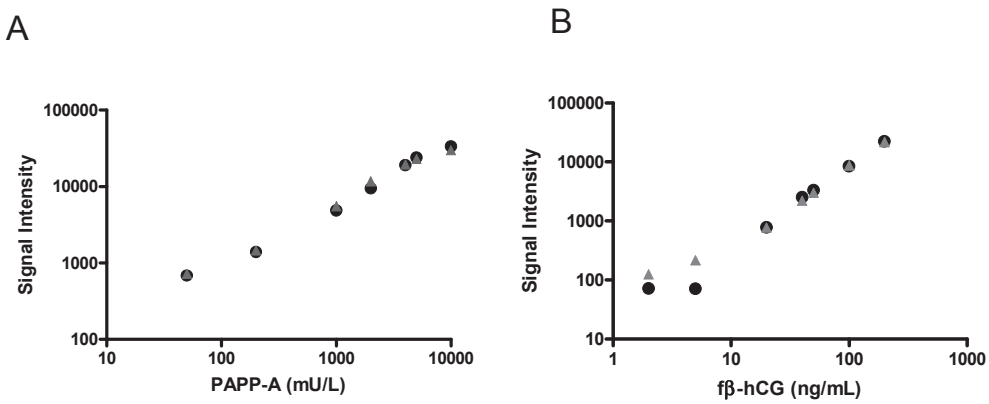


Figure 4 – The dynamic range of monoplex (black dots) and multiplex (grey triangles) indirect Antibody arrays using standards of PAPP-A (A) and fβ-hCG (B). In the multiplex arrays, a cocktail of anti-PAPP-A and anti-fβ-hCG antibodies was used as the detection antibody mixture, and as a combined standard containing recombinant PAPP-A and fβ-hCG. Values were plotted against known AutoDelfia concentrations.

The aim of the present study was to evaluate quantitative performance of Ab-arrays that allow for parallel assessment of the current Down syndrome screening markers, PAPP-A and fβ-hCG. We hypothesized that if the two current screening markers could be determined in a quantitative manner, this technique would be the obvious choice to extend the current screening with more, sensitive markers. To accomplish the evaluation, we focused on the development, performance and quality of two different Ab-array methods (direct and indirect) for the quantitative profiling of human serum markers. The advantage of the direct method is the possibility to expand an Ab-array up to thousands of antibodies, however, there are concerns that the rather large size of the dyes influences the antibody-antigen binding [314]. The direct method allows for the detection of proteins, but often fails quantitative performance and is therefore only useful in tests that require detection of the absence or presence of a protein. An explanation for the non-quantitative performance of the direct method is the labelling of serum, where steric hindrance affects labelling efficiency and

binding to the corresponding antibody, due to the relatively large dye size. Although it has been shown that quantitative performance with the direct method is possible, sandwich immunoassays (the indirect method) are mostly best-performing [308]. However, detection of multiple proteins with an indirect Ab-array requires a cocktail of detection antibodies, which limits the number of markers that can be analyzed, compared to the direct method. This is partly because including a larger number of antibodies in one assay increases the possibility of cross-reactivity, but also because antibodies need to be applicable in the same serum dilution range.

In our study we analyzed both methods under optimized array conditions. Indeed, we found that the direct method showed presence of both PAPP-A and fβ-hCG in serum, but failed to reflect quantitative levels. Consequently, the indirect Ab-array method was used for further experiments. For binding of the capture-antibody to the target, the most optimal spotted antibody concentration was determined. Interestingly, increasing concentrations of the capture-antibody strengthened the spot signal until a certain cut-off point, after which the spot signal started to decrease again. This is likely due to the fact that the amount of target becomes a limiting factor, or steric hindrance prevents optimal capture-antibody to target binding. The indirect method showed good qualitative and quantitative performance. The variation between arrays, slides and batches was within FDA-set values [312], and automation of assay execution is expected to bring the variation down to levels comparable to current automated test systems.

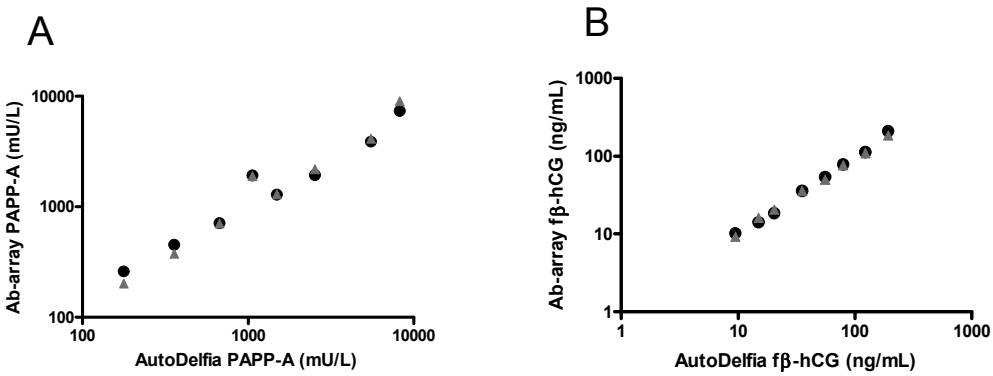


Figure 5 – Concentrations of PAPP-A (A) and fβ-hCG (B) identified using monoplex (black dots) and multiplex (grey triangles) indirect Antibody arrays. In the multiplex arrays, a cocktail of α-PAPP-A and α-fβ-hCG antibodies was used as the detection antibody mixture. Antibody array concentrations were calculated using standard curves on the same platform; values were plotted against observed AutoDelfia concentrations.

The aim of developing our Ab-array was to quantitatively determine levels of PAPP-A and fβ-hCG in serum of pregnant women, available through the screening serum bank. In order

to perform quantitative measurements of proteins, the linearity of the system is important. The dynamic range appeared linear over the occurring concentrations of both PAPP-A and fβ-hCG ranging from the 1st percentile to the 99th percentile serum concentrations available in our serum bank. Analysis of serum samples from pregnant women allowed the selection of the full dynamic range of current screening markers PAPP-A and fβ-hCG and could, therefore, be used for Down syndrome screening purposes.

Multiplex analysis of the current markers, and in the future possibly also additional markers, is a great advantage of the Ab-array method. In the current ELISA based assay, serum dilutions used to assess PAPP-A and fβ-hCG levels differ approximately 10 times. The Ab-array method allowed both proteins to be measured in the same serum dilution and with adequate performance over the entire dynamic range. The increased sensitivity of the Ab-array method over the ELISA based method could be explained by either the increased sensitivity of the dense spotted capture-antibody compared to the density in ELISA wells [307], or it could be due to the increase in fluorescent signal by the use of the Cy5 dye.

Highly interesting for its future application in a screening setting, is that small serum volumes (~10 µL) are needed for Ab-array-based analysis. As a consequence, Ab-arrays allow for collection of blood through a finger-prick and, therefore, the possibility to even implement this test in a home-setting by using dried bloodspots on filter paper, providing for very low degradation of proteins. Moreover, the screening would thereby be less invasive for pregnant women and simplifies the logistic procedures of the screening. However, one has to keep in mind that, before the indirect Ab-array method can be incorporated, further automation and validation on large study cohorts is required.

Conclusion

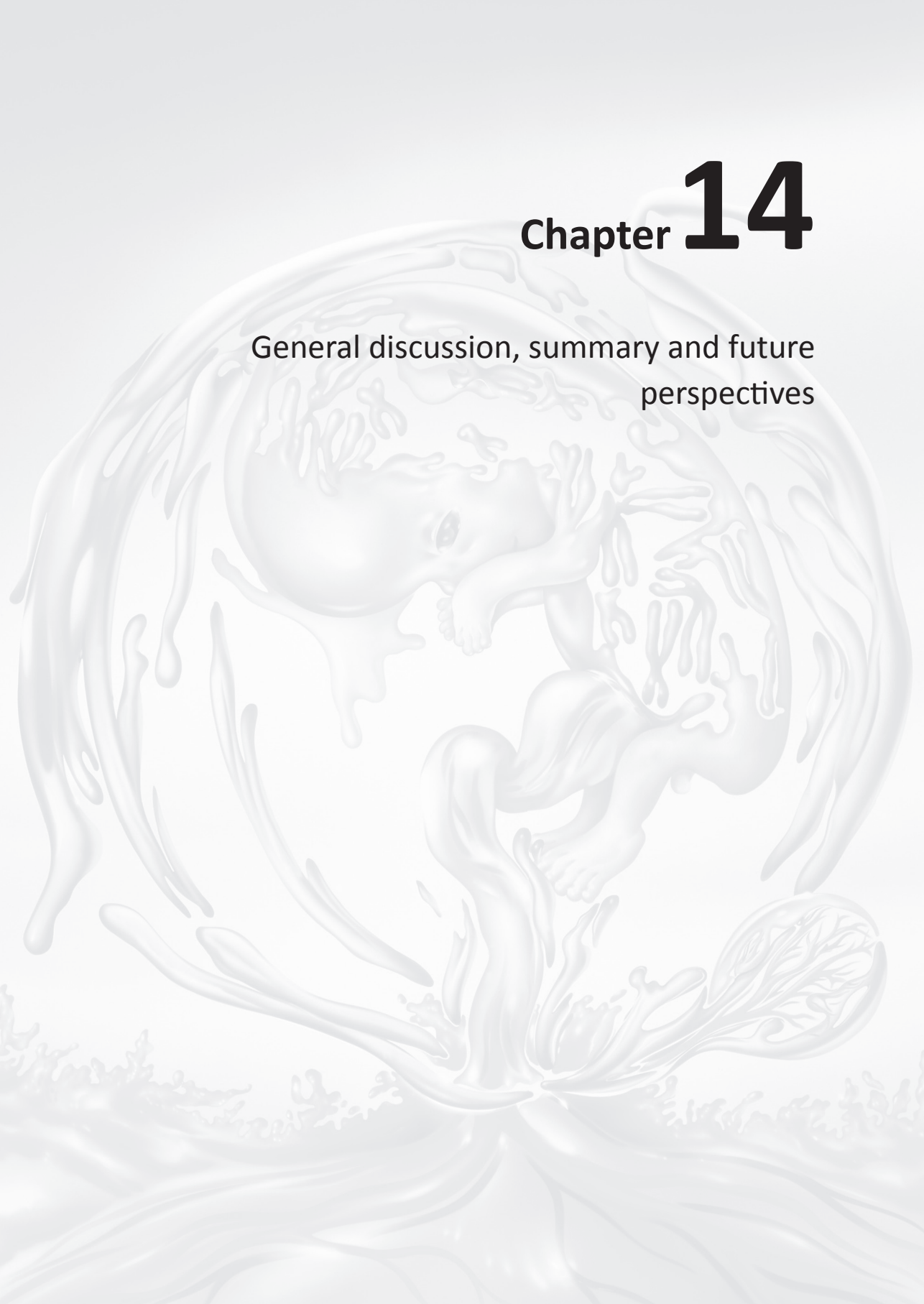
In this study we showed that currently used maternal serum markers (PAPP-A and fβ-hCG) can be quantitatively detected jointly within one assay using small amounts (~10 µL) of serum using Antibody arrays. This now opens possibilities to extend the Ab-array with new markers to improve the performance of the screening test, since the detection rate of the currently used Down syndrome screening test is rather low. For this, we are now in the process of including newly identified, promising markers in the platform to improve the detection rate of Down syndrome pregnancies [151].

Acknowledgments

We would like to thank Mark Jonker and Idder Belmouden for practical support, and José Ferreira for fitting the protein standard curve model.

Chapter 14

General discussion, summary and future perspectives



Introduction

Down syndrome (DS) is the most common chromosomal abnormality, with an incidence of approximately 1 per 500 to 800 live births [57]. DS is often associated with an impairment of cognitive ability and physical growth, and a particular set of facial characteristics. Moreover, 50% of all people with DS suffer from a congenital heart defect and DS patients are more prone to develop serious illnesses such as Alzheimer's disease, leukaemia and epilepsy. These factors all contribute to a shorter life expectancy.

The discovery of a trisomy of chromosome 21 as the underlying cause for DS and the possibility to perform a chromosome analysis on amniotic fluid allowed for the prenatal diagnosis of DS [18]. Considering the costs of amniocentesis and its accompanying risk of miscarriage, prenatal diagnosis could not be eligible for all pregnant women. Therefore, non-invasive prenatal screening became of increasing interest. Currently, the first-trimester combined test is mostly used for the prenatal prediction of carrying a child with DS. The test is composed of the maternal serum parameters pregnancy-associated plasma protein A (PAPP-A) and the free beta subunit of human chorionic gonadotropin (fβ-hCG), and an ultrasound measurement of the foetal nuchal translucency (NT), combined with maternal age.

In the Netherlands, all pregnant women are offered such prenatal screening for DS, but the uptake of the test is only 23% [315]. The Dutch prenatal screening programme is focused on extensive counselling of the pregnant woman. Women are informed about DS, the possibilities of the screening test and the consequences of high risk test results and invasive diagnostics. This way, most women are able to make a well-considered choice concerning prenatal screening. There are several reasons not to opt for prenatal screening. Firstly, some women would never terminate their pregnancy and therefore see no point in taking a screening test. Secondly, when women are under 36 years of age the screening test will usually not be reimbursed and some women are not willing to pay for the test themselves. Or thirdly, some parents do not consider having a child with DS as a heavy burden. These are all plausible arguments not to undergo prenatal screening for DS. However, another frequently heard argument is that the screening test does not provide enough certainty about the risk of having a child with DS. The detection rate (DR) of DS screening in the Netherlands is currently 76% [54], which is rather low as compared to other countries.

The research described in this thesis was performed at the Dutch National Institute for Public Health and the Environment (RIVM). The RIVM acts as the reference laboratory for DS screening in the Netherlands and processes over 10,000 first-trimester combined test per year. The RIVM therefore possesses an extensive collection of sera of pregnant women carrying a foetus with DS, trisomy 18, trisomy 13 or other congenital abnormalities. For the studies in this thesis, serum samples from this large database were used. The aim of our

research was to investigate ways to improve the performance of the current DS screening programme and, subsequently, to be able to offer all pregnant women the most accurate risk of having a child with DS.

Lacunas in current Down syndrome screening

The development of methods for DS screening has so far mainly been based on coincidences. The screening really is a spin-off of the neural tube defect (NTD) screening, and the most effective markers were discovered by fishing expeditions, not by thorough analysis of the causal relationship of genes on chromosome 21 and foetal or placental proteins that are likely to cause an excess or shortage in maternal serum as a result.

The extra chromosome in DS not only leads to anomalies of the foetus, but also of the placenta. In human trophoblast cells, the excess of oxygen radicals produced during oxygen metabolism are eliminated by natural antioxidants and superoxide dismutase (SOD). The gene responsible for this reaction is Zn-SOD and is encoded by chromosome 21. SOD expression and protein levels and activity are significantly higher (about 50%) in trophoblast cells from DS placentas. Over-expression of SOD hampers normal trophoblast formation; DS trophoblast cells cannot fully compensate for the reduced oxidative stress resulting in placental abnormalities (**chapter 2**). DS placentas show signs of impaired differentiation, aggregation and fusion of their trophoblast cells. This could lead to undervascularisation, hypotrophy and cell apoptosis of the placenta already in the first trimester of pregnancy.

The inability of placental cells to develop properly is associated with a decrease of trophoblastic products, such as hormones, proteins and growth factors. PAPP-A and fβ-hCG, currently used as DS screening markers in the first-trimester combined test, are such products. PAPP-A is a protein which is thought to be an important regulator of IGF bioavailability and cell growth [96] and fβ-hCG is a subunit of total hCG, which is the most important hormone involved in early pregnancy and provides for the maintenance of the corpus luteum and of pregnancy [316].

In normal pregnancy, serum PAPP-A concentrations increase throughout gestation more or less proportional to the size of the placenta. On the other hand, fβ-hCG concentrations slowly decrease in the first trimester. Since the concentrations do not remain constant, the risk estimation for DS uses standardized values based on gestational age (GA). Therefore, the precise determination of GA is essential. GA can be based either on the first day of the last menstrual period or based on ultrasound measurement of the crown-rump length (CRL). The latter, if measured correctly, has been shown to produce a more reliable estimate of the GA [168]. Between 2005 and 2006 health care professionals in the Netherlands based the calculation of GA on CRL in approximately two third of the pregnancies. However, it turned out that different reference curves were used to convert CRL into GA (**chapter 3**). Thus,

standardization of GA determination for first-trimester screening tests is insufficient. In the risk estimation for DS of an individual pregnancy, an incorrect GA may lead to an erroneous high risk or non-high risk outcome of the test. To overcome this, it may be considered not to convert CRL into GA, but to directly relate PAPP-A and fβ-hCG serum concentrations to CRL. Another way of improving the performance of the first-trimester combined test is to optimize the quality of the ultrasound NT measurement. Before the start of the national DS screening programme in 2007 the majority of NT measurements was of moderate quality. The average NT measurement was below the reference curve and many sonographers were not officially certified to perform NT measurements (**chapter 4**). In 2006, national quality demands for prenatal screening were set up stating that sonographers should be officially accredited to perform NT measurements and that they should perform at least 150 NT measurements a year [175]. Moreover, a new reference curve, which fits the Dutch population better, was brought into use [185]. Regional centres for prenatal screening started to actively monitor the quality of NT measurements, by both quantitative as well as qualitative evaluation of sonographers. This way, the quality of NT measurements became much more sufficient, leading to a better performance of the first-trimester combined test.

The NT measurement should be carried out between 11 and 13 weeks of gestation. A blood sample however can be taken from 8 to 13 weeks of gestation. Because of logistic reasons, most prenatal screening centres and hospitals prefer to complete the combined test in one visit; thus in the second half of the first trimester. However, there is a tendency of a higher DR and a lower false positive rate (FPR) when the collection of serum takes place before 11 weeks of gestation (**chapter 5**).

International studies propose that, for an accurate determination of the DS risk, serum marker concentrations should be corrected for smoking, ethnicity and IVF pregnancies [254, 255, 317]. However, correction factors are hard to establish, especially for smoking and ethnicity, because information is not always reliable. In the Netherlands, such correction factors are currently not used, but further research should show whether this would indeed increase the test performance.

So, if GA is determined accurately, the serum sample is taken early in the first trimester and the NT measurement is of sufficient quality the DR of the first trimester combined test will increase with a few percent. Obviously, high quality is of great importance for a screening programme but, with a risk calculation that is complicated and depends on many parameters besides the screening markers, a few percent might not be worth the effort. Therefore the question remains: how to achieve further improvement?

Application of current and suggested prenatal screening markers

In recent years, the focus of prenatal screening has expanded. Several studies have been performed to evaluate the potential of prenatal screening for foetal chromosomal abnormalities other than DS, in particular Edwards syndrome (trisomy 18) and Patau syndrome (trisomy 13). With the first-trimester combined test it is possible to detect these trisomies using the same algorithm as for DS screening [199, 200]. This way, 60% of all trisomy 18 and 13 cases will be detected through DS screening. In trisomy 18 and 13 pregnancies PAPP-A levels are decreased to a greater extent as in DS and the NT is often very large. However, oppositely to DS, serum concentrations of fβ-hCG are decreased in trisomy 18 and 13 pregnancies. Thus, by making a slight adjustment of the DS risk calculation, it would be possible to provide separate risks specifically for trisomy 18 and 13. This would lead to the detection of at least 80% of all trisomy 18 and 13 cases with only 0.2% extra false positives (**chapter 6**). The Dutch governmental license for prenatal screening was strictly confined to DS and therefore it was not allowed to report risks for trisomy 18 and 13. However, since trisomy 18 and 13 are associated with pregnancy complications and because there may be a difference in parental acceptance of a trisomy 18 or 13 pregnancy, as opposed to DS, it was decided to file a request to extend the governmental license. This request has recently been approved by the Dutch Health Council [318] and screening for trisomy 18 and 13 using a specific algorithm will probably be implemented soon.

Besides chromosomal abnormalities, first-trimester prenatal screening can also be put into use for pregnancy complications such as pre-eclampsia (PE), intrauterine growth restriction and foetal death. PE is a serious complication of pregnancy that affects approximately 1-2% of all pregnant women and it is the leading cause of maternal and perinatal morbidity and mortality [319]. The exact pathogenesis of PE remains unclear, but it is thought to result from an imbalance between placental factors, maternal constitution and unfavourable adaptive changes to pregnancy [320, 321]. Because of the serious health consequences of PE, risk assessment for PE is highly recommended. Early identification of women at risk might facilitate better antenatal surveillance, timely intervention and better outcomes. Several maternal serum markers have been investigated and found to be potentially useful as predictors of PE [214, 222, 253, 322]. One of those markers is placental protein 13 (PP13) which plays an important role in the implantation and modelling of foetal-maternal blood spaces between placenta and endometrium. PP13 is produced by the placenta, which is hampered in trisomic pregnancies, and was found to be decreased in DS pregnancies and, to greater extent, in trisomy 18 and 13 pregnancies (**chapter 7)**. Next to PP13, more placental products have been suggested as first-trimester screening markers for aneuploidies. Serum concentrations of a disintegrin and metalloprotease 12 (ADAM12) and placental growth factor (PlGF) are decreased in DS pregnancies [150, 155]. Total hCG (thCG), which is a screening

marker for DS in the second trimester of pregnancy, is increased in maternal serum from first-trimester DS pregnancies [225]. However, when these four markers are added to the current first-trimester combined test the DR increases with only 3% (**chapter 8**). It turns out that the predictive power of maternal serum markers is not constant during the first trimester. For three markers (PAPP-A, ADAM12 and PP13) the difference between DS and unaffected pregnancies is more distinct early in the first trimester (before 11 weeks), while for the remaining markers (fβ-hCG, thCG and PlGF) the difference is more pronounced later on (after 11 weeks). Based on this knowledge, it would be useful to draw two separate blood samples to increase the DR of first-trimester screening to almost 90% at a 5% FPR, which is obviously a tremendous improvement compared to the DR of the current screening program. On the other hand, adding new markers to the screening test and taking an extra blood sample bears extra costs and complicates the logistic process of first-trimester screening. A cost-effectiveness analysis is therefore necessary to evaluate the potential of such a two-sample first-trimester screening setting.

Another challenge concerning prenatal screening involves twin pregnancies. The incidence of twin pregnancies is increasing due to the advanced maternal age of pregnant women and the introduction of assisted reproductive technology [323]. Since 2009, risk estimation for DS using the first-trimester combined test in twin pregnancies is carried out routinely in the Netherlands. Screening for DS in twin pregnancies is advisable, but complicated by several factors, such as the distinction between monochorionic and dichorionic twins. Furthermore, biochemical markers give information about the whole pregnancy and not on the individual foetus. It seems that in twin pregnancies PAPP-A and fβ-hCG concentrations are, on average, twice as high compared to singleton pregnancies. Strikingly, concentrations of ADAM12 and PP13 in twin pregnancies are only approximately 1.5 times increased. The reason for this phenomenon should be further investigated if these two markers are indeed to be added to the first-trimester screening test. For none of the biochemical markers a difference in concentrations was observed between monochorionic and dichorionic twin pregnancies (**chapter 9**).

So, now there is a national screening programme which includes first-trimester screening for DS in both singleton as well as twin pregnancies and soon the screening will likely be applicable for trisomy 18 and 13 also. Four suggested first-trimester screening markers have the potential to increase the screening performance, especially when they are used in a two-sample screening setting. However, these adaptations increase the expenses of the screening program. Are there any alternatives?

Proteomics techniques to identify new screening markers for Down syndrome

A proteome is the entire complement of proteins including the modifications made to a particular set of proteins, produced by an organism or system. Proteomics is the field of research that aims at examination of the proteome in a certain tissue, cell type or body fluid at a certain time point. A plethora of emerging methodological tools allows for the study of proteins, e.g. their quantity, cellular location and post-translational modifications. Understanding the proteome, the structure and function of each protein, and the complexities of protein-interactions during a DS pregnancy may help in the search for additional biomarkers for current first-trimester DS screening.
The proteomics research in this thesis describes three phases (*Figure 1*): *i)* the discovery of new biomarkers for first-trimester DS screening, *ii)* the feasibility and validation of proteomics techniques to analyze multiple markers simultaneously, *iii)* the implementation of a cost-effective assay for large-scale screening programmes.

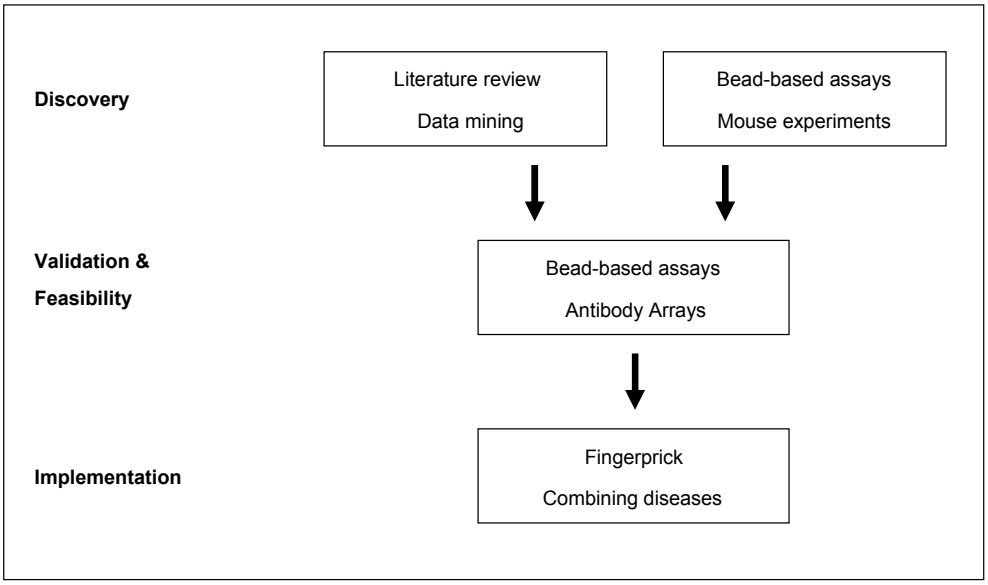


Figure 1 – Three phases of proteomics research for DS screening described in this thesis.

The presence of an extra chromosome in DS might cause deregulation and/or differential expression of proteins, e.g. cytokines and growth factors, involved in implantation and placental development and leading to their developmental disturbance [64, 65]. This may cause an increased or decreased placental expression of biological markers (hormones and proteins). If this differential expression is traceable in maternal blood these markers could

have potential as new screening markers.

In the discovery phase, an extensive review of the literature was carried out to study normal placental development and function during early pregnancy. Using this knowledge, candidate biomarkers were proposed which may be useful in screening for DS (**chapter 2**). Current screening markers for DS indeed mainly originate from the placenta, but can also be traced to the foetal liver, e.g. alpha-fetoprotein (AFP). It is therefore hypothesized that new screening markers may also originate from these tissues. However, a prerequisite of a good screening marker is that concentrations of a protein are detectable in maternal serum. The amount of information on genes and proteins in public databases is increasing rapidly, which allows for a bioinformatics approach that involves automated collection and combination of information from biological databases, known as data mining. A bioinformatics approach was developed to use data from the literature on genes and protein expression and data-textmining tools. This way, a list of 49 potential DS screening markers was generated (**chapter 10**). The list included three biomarkers that are already used for DS screening and several others, among which PP13 and PlGF, which have been examined as potential biomarkers before. Furthermore, there was a large overlap between the proposed screening markers based on the literature review and the data mining.

A more experimental proteomics approach was carried out by analyzing 90 different proteins from a pre-existing non-pregnancy-specific bead-based multiplexed immunoassay. By comparing the protein concentrations in a small cohort of DS and control sera, seven potential screening markers were identified (**chapter 11**). None of the identified proteins is linked to genes located on chromosome 21. However, some of the markers are known to be highly expressed in the placenta or foetal liver and were also proposed in the candidate biomarker lists from the previously described discovery studies. Unfortunately, none of the seven identified markers showed major differences between cases and controls. It might be that biomarkers with large distinctive power were not present on the immunoassay or, alternatively, that fold changes are inherently not high in maternal blood. Nevertheless, the addition of the seven biomarkers to the current screening test provided a significant improvement of the detection rate for DS. Despite these promising results, it is obvious that test performance is always better when a screening test is applied to the same cases from which the markers are derived and therefore application of the proposed markers on a different cohort of cases is essential to establish the true diagnostic accuracy of the immunoassay. This was done in a subsequent validation study in which 34 DS cases and matching controls were included to confirm the predictive value of the seven markers found in the discovery study. Epidermal growth factor (EGF) and EN-RAGE were confirmed to be potential screening markers for DS and improved the DR of the current first-trimester combined test with approximately 6% (**chapter 12**). This may seem rather disappointing considering the initial identification of seven potential markers. On the other hand, the

finding that two markers again improved the DS screening performance in an independent study is highly encouraging. In addition to the two validated markers, Cancer Antigen 19-9 (CA19-9), which was not distinctive between DS and controls in the discovery study, was now found to be strongly predictive for DS and even further increased the DR. Clearly, large scale validation experiments need to be performed to provide sufficient evidence for potential markers before they can be implemented in a screening test.

If an extended screening test is to be developed, in which several biochemical markers are included, simultaneous assessment of markers is crucial. The current first-trimester combined test is based on enzyme-linked immunosorbent assay (ELISA) methods, which is widely used for quantitative protein measurements. ELISA is a reproducible and specific assay, but lacks the capacity of simultaneous assessment of multiple markers. The experiments carried out to identify and validate new markers were performed using bead-based multiplexed immunoassays. Such assays use colour-coded tiny beads in up to 100 distinct sets. Coating each bead set with a specific reagent allows the capture and detection of many specific analytes, such as proteins, from a sample. Next, labelled beads are incubated with serum samples and, subsequently, with a detection antibody labelled with a reporter dye in a bead-based immunoassay. Flow cytometry equipment measures the internal dyes to identify each particle and the reporter dye captured during the assay [324]. The technology allows multiplexing many unique markers within a single sample, both rapidly and precisely, in a high-throughput setting.

Another method allowing for the analysis of many markers simultaneously is the use of Antibody microarrays (Ab-arrays). Ab-arrays are a platform for protein expression profiling. Small amounts of capture antibodies for the selected targets are immobilized or spotted on a very small area on coated glass slides. The high density of the capture antibodies in the spots that is obtained enables high sensitivity [307]. Analysis of serum samples from pregnant women allowed the selection of the full dynamic range of current screening markers PAPP-A and fβ-hCG. The indirect method showed good qualitative and quantitative performance compared to the initial ELISA measurements (**chapter 13**). Since all samples were accurately quantified using this technique, the feasibility of Ab-arrays within the framework of first-trimester screening for DS is excellent.

Future perspectives and ongoing research

For the optimization of the current DS screening programme there is still a lot to gain in the ‘old school’ approach. That approach focuses on the use of current markers in a more sophisticated way: by applying correction factors for smoking, ethnicity and IVF pregnancies in the DS screening algorithm; by introducing additional non-invasive tests, such as extensive ultrasound examinations, for those who have a high risk at an initial biochemical

test (contingent screening); or by measuring markers at two different time points in the first trimester, to be able to maximize the distinctive power of each marker.

Currently, knowledge to do in-depth evaluations based on genomic, proteomic and transciptomic techniques is available. One line of research concerning DS screening tries to put high-end quantification techniques for DNA and RNA into use to quantify foetal DNA or RNA, either in nuclei of foetal cells or free-floating in maternal serum [325]. With this promising non-invasive technique, it may be possible to provide definite identification of DS. However, there are still many limitations to the technique. The abundance of foetal DNA and RNA in maternal blood is very low and detection methods are expensive. Furthermore, the technique is not feasible for every pregnant woman because it is not always possible to discriminate between foetal and maternal DNA/RNA [63]. New techniques, such as shotgun sequencing [61], are being developed to cover these issues, but it is still a long way from implementation in a high-throughput screening setting.

The research described in this thesis focuses on the more directive search for new markers for DS using the ever expanding knowledge of the human genome and proteome and combines both laboratory techniques and digital evaluation of data (data mining). Recently, two-dimensional gel electrophoresis (2-D), tandem mass spectrometry (MS-MS) and bead-based multiplexed immunoassays have been used to identify several potential biomarkers in amniotic fluid and maternal blood [257, 258, 260, 291], clearly demonstrating the potential of applying proteomics techniques in the quest for new biomarkers. In the boost of new development the question arises whether these advanced detection techniques will be available at a reasonable cost, a prerequisite for screening tests. In principle however, these new techniques are calculated to cost within the range of 20-50 euros per screening.

Ongoing research of the proteomics project described in this thesis also includes mouse-models for biomarker identification. Breeding healthy female mice and male transgenic mice with DS (type Ts(16C-tel)1Cje; The Jackson Laboratory, Bar Harbor, ME, USA) produces healthy females pregnant with, on average, 50% DS embryos. Blood is drawn from the pregnant mice, three times during the first trimester, for the identification of potential screening markers in maternal serum. Then, the pregnancy is terminated and the placenta, the foetal liver and the foetal heart are collected. Both gene and protein profiles will be analyzed to study the difference between DS and unaffected siblings. Genes and proteins that show over- or underexpression in DS foetuses can be compared to those detected in maternal serum and should be considered potential biomarkers.

While most of the research is focused on the screening for DS, it is not unthinkable that a number of other diseases (apart from trisomy 18 and 13) can be screened for. It must surely be possible to detect, in the same samples, other parameters of prenatal screening (e.g. irregular blood types and infectious diseases, like HIV and hepatitis) and to identify high risks for foetal and maternal pregnancy complications. This spin-off of the current proteomics

project may have opened a completely new field of research. Currently, a similar approach has been set up to identify potential screening markers for pre-eclampsia, a rather common and serious complication in pregnant women.
Especially new proteomic techniques will need only minute amounts of test material; 10-20 micro-litres serum instead of 1-2 ml. Hypothetically, this downscaling opens the possibility to draw small amounts of blood and to replace the relatively laborious venous puncture with a finger-prick, possibly carried out in the home situation, and sent to the laboratory on filter paper in the mail (providing for very low degradation of proteins). This is possibly needed to easily arrange for two blood samples taken at different time points in the first trimester. The laboratory will be able to analyze a combination of approximately ten markers. In combination with sophisticated algorithms this may lead to a screening test, to be carried out by the pregnant women herself, giving her a reassuring high detection rate for several diseases and pregnancy complications.
In the next coming years, the outline of such a future prenatal screening is feasible, however; it will probably take some time before these methods can be tested in large cohorts that proof their efficacy as a screening tool, a bare necessity before actual implementation can take place.

Conclusions and recommendations

- The current screening for Down syndrome in the Netherlands can be improved by accurately measuring the crown-rump length of the foetus for the determination of gestational age, by improving the quality of nuchal translucency (NT) measurements through monitoring and training of sonographers and by drawing the serum sample early in the first trimester (before 11 weeks).

- By slightly adjusting the algorithm for Down syndrome screening it is possible to identify approximately 80% of foetuses with Edwards syndrome (trisomy 18) or Patau syndrome (trisomy 13), with only 0.2% extra false positive test results.

- Because of the increasing incidence of twin pregnancies, screening for aneuploidies should not be limited to singleton pregnancies. By adjusting the biochemical screening markers it is possible to perform Down syndrome screening in twin pregnancies without having to distinguish between monochorionic and dichorionic twins.

- To increase the Down syndrome screening performance it would be advisable to draw two serum samples during the first trimester. One sample before 11 weeks to measure PAPP-A and one sample after 11 weeks to measure fβ-hCG. At these points in time, the

biochemical screening markers are most distinctive between Down syndrome and unaffected pregnancies. Logistically it would be best to draw the second serum sample at the time of the NT measurement.

- Adding potential biochemical screening markers, such as ADAM12, PP13, PlGF and thCG, to the first-trimester combined test increases the detection rate for aneuploidies, but also allows screening for other pregnancy complications.

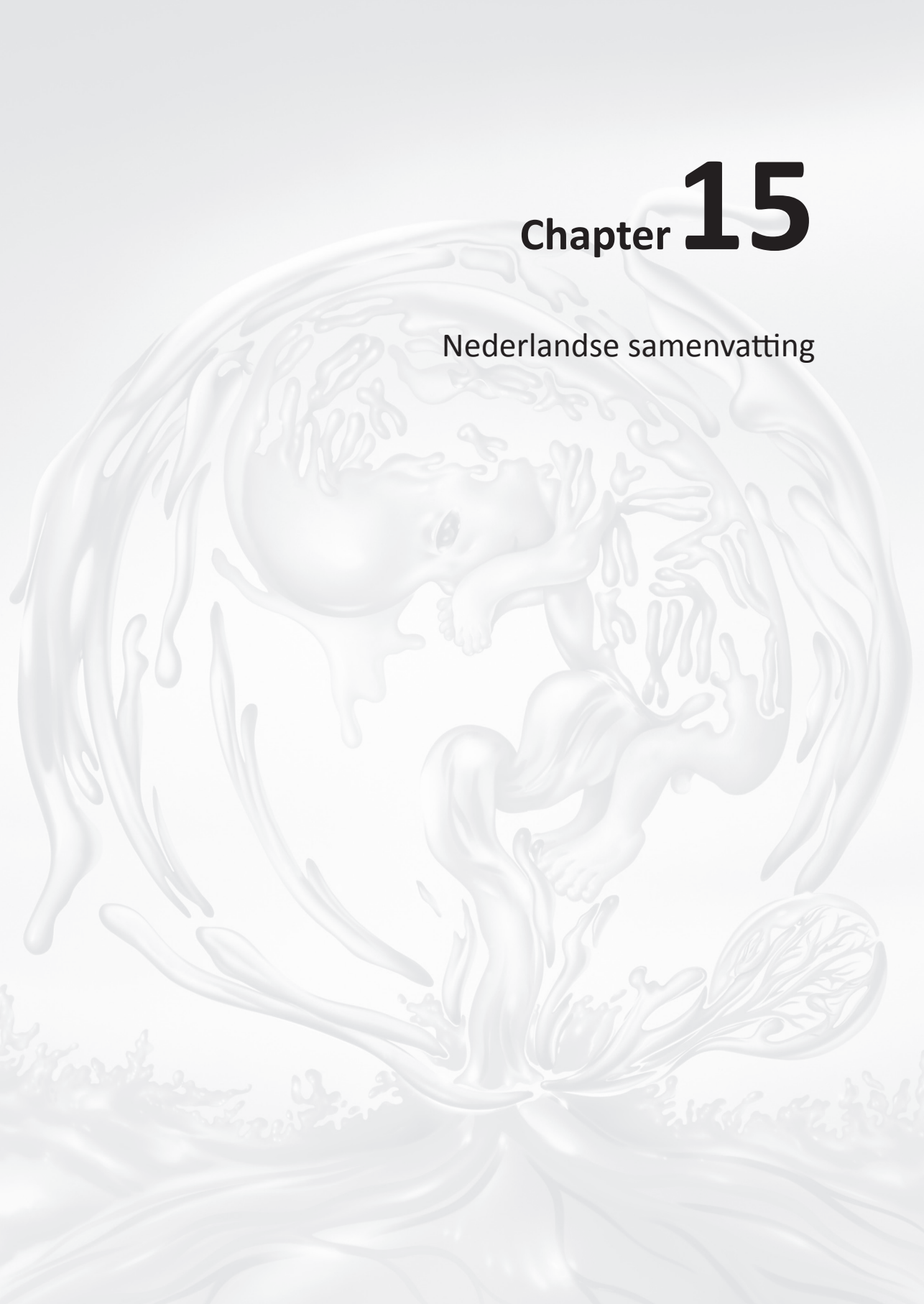
- Proteomics techniques are applicable for the identification of new biochemical screening markers for Down syndrome. EGF, EN-RAGE and CA19-9 are proposed as potential screening markers, but need to be tested in large-scale validation experiments.

- Bead-based multiplexed immunoassays and Antibody microarrays are high-throughput platforms that allow for multiple marker analyses. Therefore, these techniques can be used for Down syndrome screening purposes in the future.

- Future prenatal screening will probably focus on several foetal abnormalities and maternal complications using a panel of biochemical markers, which can be analyzed simultaneously, in one screening test.

Chapter 15

Nederlandse samenvatting



In het kort

In Nederland heeft iedere zwangere vrouw de mogelijkheid om screening op Downsyndroom te laten uitvoeren. Dit gebeurt door middel van een risicoschatting waarbij de leeftijd van de moeder, de concentraties van twee markers in het bloed van de moeder en de dikte van de nekplooi van het ongeboren kind worden meegenomen. Op deze manier worden in Nederland 70-75% van alle Downsyndroom zwangerschappen opgespoord. Deze screening kan echter op relatief eenvoudige wijze worden verbeterd. Ten eerste door niet één maar twee keer vroeg in de zwangerschap bloed van de zwangere af te nemen; één keer vóór de 11^{e} week van de zwangerschap en één keer tussen de 11^{e} en 14^{e} week. Ten tweede zouden er meerdere markers in het bloed van de moeder kunnen worden bepaald. Dergelijke aanpassingen brengen het aantal opgespoorde Downsyndroom zwangerschappen naar ongeveer 90%. Een bijkomend voordeel van de nieuwe markers is dat ze ook gebruikt kunnen worden voor het opsporen van andere aangeboren aandoeningen en ernstige zwangerschapscomplicaties, zoals zwangerschapsvergiftiging. Nieuwe laboratoriumtechnieken maken het mogelijk meerdere markers tegelijkertijd te bepalen tegen lage kosten. Kortom, deze ontwikkelingen openen een scala aan nieuwe mogelijkheden op het gebied van vroege zwangerschapsscreening.

Introductie

Down syndroom (DS) is de meest voorkomende chromosomale afwijking en komt voor bij ongeveer 1 op de 500 tot 800 levend geboren kinderen [57]. DS is vaak geassocieerd met cognitieve en fysieke afwijkingen, waaronder specifieke gezichtskenmerken. Daarnaast heeft 50% van alle mensen met DS een aangeboren hartafwijking en komen ernstige ziekten, zoals Alzheimer, leukemie en epilepsie, vaak voor. DS patiënten hebben een kortere levensverwachting.

De ontdekking van drie kopieën (trisomie) van chromosoom 21 als de oorzaak van DS en de mogelijkheid om chromosomaal onderzoek in vruchtwater uit te voeren, hebben het mogelijk gemaakt om DS al vroeg in de zwangerschap op te sporen [18]. Een diagnostische vruchtwaterpunctie of vlokkentest geeft echter kans op een miskraam en de kosten van dit onderzoek zijn hoog. Daarom is het goed om zwangeren met een hoog risico op het dragen van een foetus met Downsyndroom te identificeren met een niet-invasieve (en daarmee veilige) prenatale screeningstest. Hiervoor wordt meestal de zogenaamde eerste-trimester combinatietest gebruikt. Bij deze test worden de concentraties van pregnancy-associated plasma proteïne A (PAPP-A) en de vrije bèta subunit van humaan chorion gonadotropine (fβ-hCG) in het bloed van de moeder bepaald. Ook wordt met een echo de dikte van de nekplooi van het ongeboren kind (foetus) gemeten. Op basis van de gemeten waarden, in combinatie met de leeftijd van de moeder, wordt het risico op het krijgen van een kind met DS berekend.

In Nederland wordt aan iedere zwangere vrouw prenatale screening op DS aangeboden. Ongeveer 23% van alle zwangeren neemt deel aan deze screening [315]. Het aantal DS zwangerschappen dat door screening wordt opgespoord (het detectiepercentage; DR) ligt in Nederland tussen de 70% en 75%, wat laag is in vergelijking met screeningsprogramma's in andere landen.

Het onderzoek in dit proefschrift werd uitgevoerd bij het Rijksinstituut voor Volksgezondheid en Milieu (RIVM). Het RIVM is referentielaboratorium voor DS screening en verwerkt ongeveer 10.000 aanvragen voor eerste-trimester combinatietesten per jaar. Het RIVM beschikt daardoor over een uitgebreide collectie bloedmonsters van vrouwen die zwanger waren van een kind met aangeboren afwijkingen, waaronder DS. Voor de studies in dit proefschrift zijn deze bloedmonsters gebruikt. Het onderzoek had drie hoofddoelen: (1) het verbeteren van de kwaliteit van de huidige DS screening, (2) het onderzoeken van de voorspellende waarde van bekende merkstoffen (markers) voor DS en andere chromosoomafwijkingen, en (3) door met behulp van nieuwe (proteomics) technieken te zoeken naar andere markers die voorspellend zijn voor DS.

Hiaten in de huidige Down syndroom screening

Het ontwikkelen van methoden voor DS screening was tot nu toe vooral gebaseerd op toevallige ontdekkingen. De meeste markers zijn min of meer bij toeval gevonden en niet door een grondige analyse van genen en eiwitten die verhoogd of verlaagd kunnen zijn in moederlijk bloed als gevolg van het extra foetale chromosoom.
De aanwezigheid van dit extra chromosoom bij DS leidt niet alleen tot afwijkingen bij het kind, maar ook in de placenta. Bij placenta's van DS zwangerschappen is sprake van een verminderde of vertraagde vorming en samensmelting van cellen. Dit kan leiden tot verslechterde doorbloeding, celdood en verminderde groei van de placenta, zeer vroeg in de zwangerschap. Het onvermogen van deze cellen om zich normaal te ontwikkelen gaat gepaard met verminderde productie van hormonen, eiwitten en groeifactoren in de placenta (**hoofdstuk 2**). Twee voorbeelden hiervan zijn PAPP-A en fβ-hCG, de markers die gebruikt worden in de huidige eerste-trimester combinatietest.
In een gezonde vroege zwangerschap neemt de productie van PAPP-A geleidelijk toe, min of meer in verhouding met de grootte van de placenta. De productie van fβ-hCG neemt juist af. Bij iedere zwangerschapsduur horen dus andere normaalwaarden van markers. Bij de eerste-trimester combinatietest wordt daar rekening mee gehouden. Daarom is het van groot belang dat de zwangerschapsduur nauwkeurig wordt bepaald. De zwangerschapsduur kan worden berekend op basis van de eerste dag van de laatste menstruatie of op basis van een echoscopische meting van de kruin-stuitlengte (CRL) van de foetus. In Nederland werd in 2005 en 2006 in ongeveer tweederde van de zwangerschappen de CRL gebruikt voor de bepaling van de zwangerschapsduur. Echter, deze bepaling was niet altijd correct, omdat de referentiecurven die gebruikt werden om een CRL om te rekenen naar zwangerschapsduur van elkaar verschilden (**hoofdstuk 3**). Dit betekent voor het berekenen van het risico op DS dat, in sommige gevallen, een onterecht hoog- of laag-risico uitslag wordt berekend. Om dit te voorkomen is het van belang om één correcte referentiecurve te gebruiken.
Een andere manier om de eerste-trimester combinatietest te verbeteren is het optimaliseren van de kwaliteit van de nekplooimeting (NT-meting). Vóór de invoering van het nationale prenatale screeningsprogramma in 2007 werd de gemiddelde nekplooi te klein gemeten (**hoofdstuk 4**). In 2006 werden, door het Centrum voor Bevolkingsonderzoek landelijke kwaliteitseisen opgesteld waarin onder andere staat dat een echoscopist minstens 150 NT-metingen per jaar moet verrichten [175]. Daarnaast werd een nieuwe, betere, referentiecurve voor NT-metingen in gebruik genomen [185] en zijn regionale centra voor prenatale screening begonnen met actieve kwaliteitsbewaking. Deze maatregelen hebben geleid tot een aanzienlijke verbetering van de kwaliteit van de NT-metingen en daarmee dus ook van de kwaliteit van de eerste-trimester combinatietest.
Een NT-meting moet worden uitgevoerd tussen de 11^{e} en de 13^{e} week van de zwangerschap,

maar het bloed kan al vanaf de 8e week worden afgenomen. Vanwege logistieke redenen kiezen veel zorgverleners ervoor om de bloedafname en de NT-meting op hetzelfde moment te laten plaatsvinden, dus na de 11e week. In dit proefschrift wordt aangetoond dat als de bloedafname vroeg in het eerste trimester zou plaatsvinden, namelijk vóór de 11e week, 84% van alle DS zwangerschappen wordt opgespoord tegenover 73% als het bloed na de 11e week wordt afgenomen (**hoofdstuk 5**).

Kortom, als de zwangerschapsduur nauwkeurig wordt bepaald, de bloedafname vroeg in het eerste trimester plaatsvindt en de NT-meting van goede kwaliteit is zal de DR van de eerste-trimester combinatietest met een paar procent verbeteren. Natuurlijk is goede kwaliteit van screening erg belangrijk, maar een paar procent is nog niet genoeg voor een optimale screeningstest. Dus, hoe kan de DS screening nog verder worden verbeterd?

Toepassing van huidige en gesuggereerde prenatale screeningsmarkers

De laatste jaren is de focus van prenatale screening verbreed. Er zijn verschillende studies verschenen waarin de mogelijkheid van prenatale screening voor andere aandoeningen dan DS is onderzocht. Het gaat daarbij met name om Edwards syndroom (trisomie 18) en Patau syndroom (trisomie 13), twee syndromen die gepaard gaan met ernstige aangeboren afwijkingen en een gemiddelde levensverwachting van slechts enkele maanden. Met de huidige eerste-trimester combinatietest worden ongeveer 60% van deze trisomiën opgespoord, maar door een kleine aanpassing te maken in de DS risicoberekening kunnen eenvoudig specifieke risico's voor trisomie 18 en 13 worden berekend [199, 200]. Deze aanpassing heeft tot gevolg dat meer dan 80% van deze trisomiën kunnen worden opgespoord (**hoofdstuk 6**). Deze resultaten zijn onderdeel geweest van de informatie op basis waarvan de Gezondheidsraad heeft geadviseerd om ook prenatale screening voor trisomie 18 en 13 in Nederland aan te bieden [318]. Recent is dit advies overgenomen door de minister van VWS.

Naast aangeboren afwijkingen kan prenatale screening ook gebruikt worden voor het opsporen van zwangerschapscomplicaties, zoals zwangerschapsvergiftiging (pre-eclampsie). Van verschillende markers is de voorspellende waarde voor pre-eclampsie onderzocht [214, 222, 253, 322]. Eén van deze markers is placental protein 13 (PP13), dat een belangrijke rol speelt in de vorming van de bloedvaten tussen de placenta en de baarmoeder. PP13 concentraties zijn verlaagd in het bloed van zwangeren met een kind met DS, trisomie 18 of trisomie 13 (**hoofdstuk 7**). Ook van andere stoffen dan P13 wordt vermoed dat ze kunnen dienen als markers voor aangeboren afwijkingen vroeg in de zwangerschap. In het bloed van vrouwen met een DS zwangerschap zijn concentraties van a disintegrin and metalloprotease 12 (ADAM12) en placental growth factor (PlGF) verlaagd [150, 155] en concentraties van totaal hCG (thCG) juist verhoogd [225]. Als deze vier markers toegevoegd worden aan de huidige eerste-

trimester combinatietest stijgt de DR van 77% naar 80% (**hoofdstuk 8**). De voorspellende waarde van deze markers is niet altijd hetzelfde, maar hangt af van de zwangerschapsduur waarbij ze worden bepaald. PAPP-A, ADAM12 en PP13 zijn meer voorspellend voor DS als ze vóór de 11e week van de zwangerschap worden bepaald en fβ-hCG, thCG en PlGF zijn juist meer voorspellend voor DS na de 11e week. Daarom zou het zinvol zijn om niet één, maar twee keer bloed af te nemen voor prenatale screening en de markers dus gespreid over het eerste trimester te meten. Dit zal de DR verder verhogen tot bijna 90%.
Doordat de gemiddelde leeftijd van zwangere vrouwen is toegenomen en er steeds meer gebruik wordt gemaakt van voortplantingstechnieken, zoals IVF, komen de laatste jaren steeds meer tweelingzwangerschappen voor [323]. In Nederland wordt de eerste-trimester combinatietest sinds 2009 ook uitgevoerd bij tweelingzwangerschappen. PAPP-A en fβ-hCG concentraties zijn ongeveer twee keer zo hoog als in éénlingzwangerschappen en de concentraties van ADAM12 en PP13 anderhalf keer zo hoog. Er werden geen verschillen in markerconcentraties gevonden tussen eeneiige en twee-eiige tweelingzwangerschappen (**hoofdstuk 9**).
Kortom, op dit moment is er in Nederland een DS screeningsprogramma voor eenling- en tweelingzwangerschappen en binnenkort zal het programma worden uitgebreid met de screening naar trisomie 18 en 13. Vier gesuggereerde markers hebben de potentie om de DR te verbeteren, vooral als er in het eerste trimester twee bloedafnamen plaatsvinden. Maar, deze veranderingen verhogen waarschijnlijk de kosten van het screeningsprogramma. Dus, zijn er alternatieven?

Proteomics technieken voor het identificeren van nieuwe screeningsmarkers voor Down syndroom

Proteomics is de studie van eiwitten (proteïnen) in weefsels en lichaamsvloeistoffen. Een scala aan technieken maakt het mogelijk deze eiwitten te bestuderen. Door inzicht te verkrijgen in de structuur en functie van eiwitten en hun interacties gedurende de zwangerschap kunnen nieuwe markers voor DS screening worden geïdentificeerd.
Het proteomics onderzoek in dit proefschrift heeft drie fasen (zie figuur 1 van hoofdstuk 14): *i)* de identificatie van nieuwe markers voor DS screening, *ii)* de toepasbaarheid en validatie van proteomics technieken, en *iii)* de implementatie van een kosteneffectieve test.
In de eerste fase van het onderzoek is een uitgebreid overzicht van de literatuur gemaakt om de ontwikkeling van de placenta in DS zwangerschappen te bestuderen (**hoofdstuk 2**). Daarnaast is met behulp van publieke databases, met uitgebreide informatie over genen en eiwitten, gezocht naar markers die geproduceerd worden door de placenta en waarvan de concentraties in bloed van een zwangere verhoogd of verlaagd zijn in DS zwangerschappen (**hoofdstuk 10**). Op basis van al deze gegevens werd een lijst met nieuwe, mogelijke

screeningsmarkers opgesteld. Sommige van deze markers worden al gebruikt voor DS screening (PAPP-A, fβ-hCG, AFP) en verschillende anderen, waaronder PP13 en PlGF, zijn eerder al gesuggereerd en onderzocht als mogelijke markers.

Een meer experimentele aanpak werd uitgevoerd door het tegelijkertijd meten van veel verschillende eiwitten in een klein aantal bloedmonsters. Van de 90 onderzochte eiwitten lieten zeven een duidelijk verschil in concentraties zien tussen DS en gezonde zwangerschappen en werden daarom gezien als mogelijke markers voor DS (**hoofdstuk 11**). Gezamenlijk konden deze markers alle 15 DS zwangerschappen onderscheiden van alle 14 gezonde zwangerschappen (DR = 100%). Om deze resultaten te valideren werd een nieuw experiment opgezet met bloedmonsters van 34 DS en 34 gezonde zwangerschappen. Van de zeven markers uit de eerste studie bleken epidermal growth factor (EGF) en EN-RAGE weer een verschil op te leveren en zijn daarom veelbelovend als screeningsmarker. Als beide markers toegevoegd zouden worden aan de huidige screeningstest stijgt de DR met ongeveer 6% (**hoofdstuk 12**). Behalve EGF en EN-RAGE bleek ook cancer antigen 19-9 (CA19-9) voorspellend te zijn voor DS. Deze marker was echter niet geïdentificeerd als screening marker in de eerste studie en moet daarom nog verder gevalideerd worden. De verschillende uitkomsten tussen het eerste en het tweede experiment impliceren dat niet elke zwangerschap met een kind met Downsyndroom een gelijk eiwitprofiel in het moederlijk bloed toont. Dit bemoeilijkt vooralsnog implementatie van een dergelijke test.

Door het toevoegen van al deze nieuwe markers aan de screeningtest wordt het noodzakelijk dat die markers tegelijkertijd in hetzelfde monster gemeten kunnen worden. De meest gebruikte methode voor het bepalen van markers is de zogenaamde ELISA. ELISA is een betrouwbare techniek voor het meten van hoeveelheden van specifieke eiwitten maar voor iedere marker heb je een afzonderlijke ELISA nodig. Voor de proteomics studies in dit proefschrift is gebruik gemaakt van bead-based multiplex immunoassays. Deze assays bestaan uit ongeveer 100 kleurgecodeerde bolletjes. Ieder bolletje wordt gekoppeld aan een specifiek antilichaam (één voor iedere marker), zodat het in principe mogelijk is om 100 verschillende markers te meten op één assay [324].

Een andere methode om meerdere markers tegelijkertijd te meten is de zogenaamde Antibody-microarray (Ab-array). Hierbij worden kleine hoeveelheden antilichamen gespot op een objectglas; één spot voor iedere marker [307]. Een experiment waarbij PAPP-A en fβ-hCG werden gemeten in bloed van zwangeren, met behulp van Ab-arrays, liet bijna dezelfde concentraties zien in vergelijking met de ELISA techniek (**hoofdstuk 13**). Dit betekent dat Ab-arrays ook een goed alternatief kunnen zijn voor het meten van markers voor DS screening.

Kortom, met behulp van proteomics technieken kunnen markers voor DS screening worden geïdentificeerd en gevalideerd. Die zoektocht heeft tot nu toe geleid tot de ontdekking van twee veelbelovende nieuwe screeningsmarkers: EGF en EN-RAGE. Om voor de DS screening meerdere markers tegelijkertijd te kunnen meten zijn bead-based multiplex immunoassays

en Ab-arrays geschikte methoden. Dus, hoe ziet de toekomstige prenatale screening er dan wellicht uit?

Toekomstperspectieven en verder onderzoek

Het onderzoek in dit proefschrift is gericht op de zoektocht naar nieuwe markers voor DS screening, gebruik makend van de kennis van genen en eiwitten. In de afgelopen jaren zijn verschillende proteomics technieken gebruikt voor het vinden van nieuwe screeningsmarkers [257, 258, 260, 291]. Gezien de grootschaligheid van het screeningsprogramma is het belangrijk dat deze technieken beschikbaar zijn tegen een redelijke prijs; de verwachting is dat een nieuwe screeningstest (bijvoorbeeld een multiplex immunoassay of Ab-array), met meerdere markers, tussen de 20 en 50 euro zal gaan kosten.
Het meeste onderzoek is gericht op het vinden van nieuwe markers voor DS. Het is echter niet ondenkbaar, en soms zelfs al mogelijk en toegestaan, dat, in dezelfde bloedmonsters, ook gescreend kan worden op andere aandoeningen, bijvoorbeeld trisomie 18 en 13, en zwangerschapscomplicaties zoals zwangerschapshypertensie. Een proteomics-benadering zoals voor DS (zowel voor het identificeren van markers als de bruikbaarheid van de technieken) kan op dezelfde wijze uitgevoerd worden voor andere aandoeningen en op dit moment werken wij aan een vergelijkbare aanpak voor vroege screening naar zwangerschapshypertensie en pre-eclampsie.
Voor een analyse met proteomics technieken is maar een zeer kleine hoeveelheid bloed nodig; 10-20 microliter in plaats van 1-2 ml. In principe zou het daarmee mogelijk worden om de normale (veneuze) bloedafname te vervangen door een vingerprik, die vervolgens als bloedvlekken op filterpapier naar het laboratorium kan worden gestuurd.
In de komende jaren is een dergelijke, futuristische prenatale screening haalbaar, maar voordat het daadwerkelijk zover komt is verder onderzoek naar de mogelijkheden en de kosten noodzakelijk.

Conclusies en aanbevelingen

- De huidige Nederlandse screening op Down syndroom kan worden verbeterd door het adequaat meten van de kruin-stuitlengte voor het bepalen van de zwangerschapsduur, door de kwaliteit van de nekplooimeting te verbeteren met behulp van strikte kwaliteitsbewaking en training van echoscopisten en door de bloedafname vroeg in het eerste trimester te laten plaatsvinden.

- Met een kleine aanpassing van het screeningsalgoritme voor Down syndroom wordt het mogelijk om 80% van alle foetussen met trisomie 18 en 13 op te sporen.

- Het is aan te bevelen om DS screening ook aan te bieden in geval van tweelingzwangerschappen. Dit is mogelijk door voor iedere marker een correctiefactor voor tweelingen in te voeren, zonder dat het nodig lijkt daarbij onderscheid te maken tussen eeneiige en twee-eiige tweelingzwangerschappen.

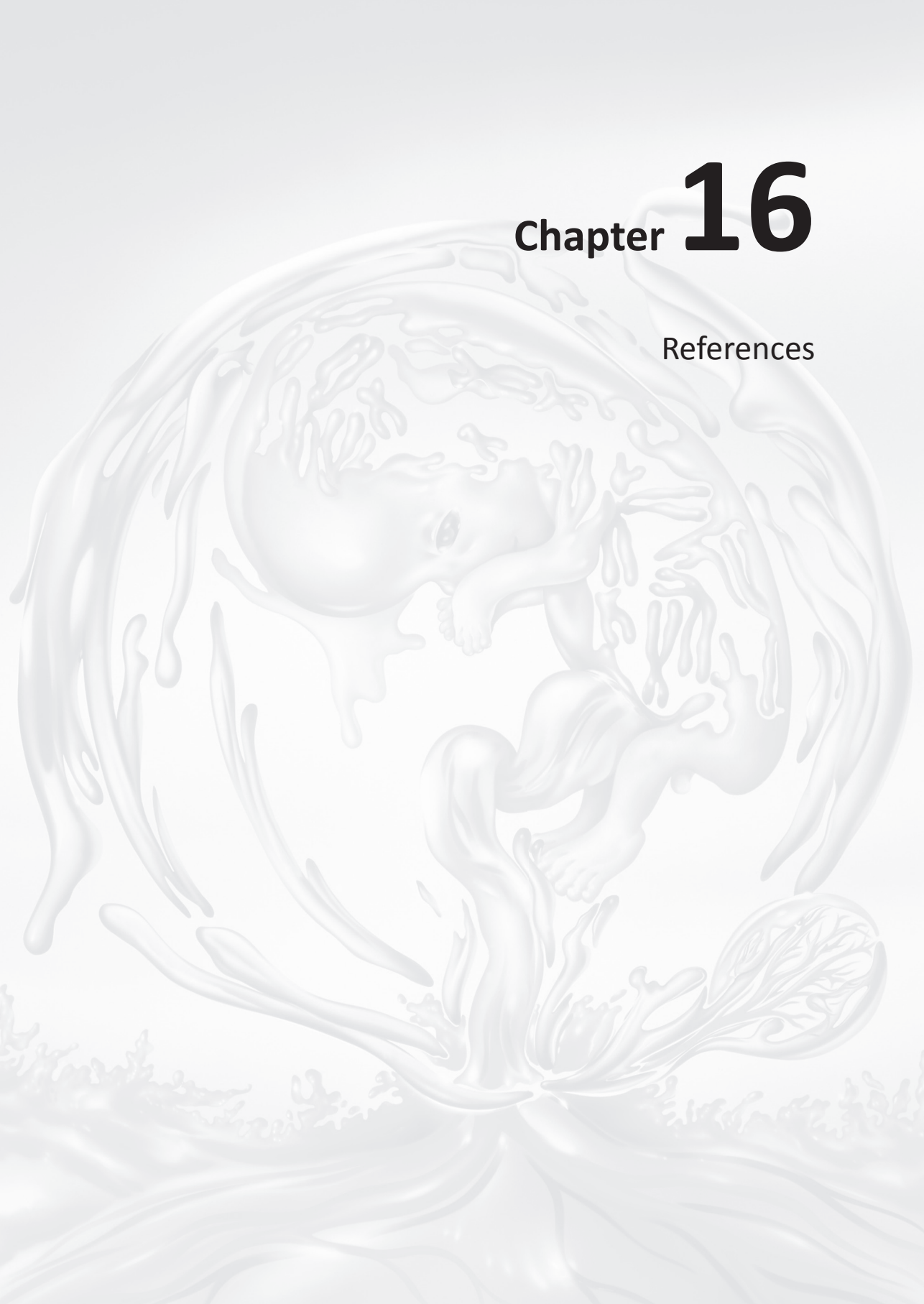
- Om het detectiepercentage voor Down syndroom te verbeteren is het aan te bevelen om vroeg in de zwangerschap twee bloedmonsters af te nemen. Eén bloedafname vóór de 11e week van de zwangerschap voor het meten van PAPP-A en één bloedafname daarna voor het meten van fβ-hCG. Vanuit logistiek oogpunt is het handig om ten tijde van de nekplooimeting de tweede bloedafname te doen.

- Het toevoegen van de screeningsmarkers ADAM12, PP13, PlGF en thCG aan de eerste-trimester combinatietest verhoogt de detectie van Down syndroom en andere zwangerschapscomplicaties.

- Proteomics technieken zijn geschikt voor het ontdekken van mogelijke screeningsmarkers voor Down syndroom. EGF, EN-RAGE en CA19-9 zijn zulke markers, maar verdere validatie in grootschalige studies is nodig voordat ze ook werkelijk als screeningsmarker ingezet kunnen worden.

- Bead-based multiplex immunoassays en Antibody-arrays zijn geschikt om snel meerdere markers tegelijkertijd te analyseren. Deze technieken zijn daarom veelbelovend voor Down syndroom screening in de toekomst.

- De toekomstige prenatale screening zal waarschijnlijk bestaan uit een test voor verschillende aandoeningen van zowel moeder als kind, waarbij meerdere screeningsmarkers worden bepaald, met meerdere testmomenten in het eerste trimester van de zwangerschap.



Chapter 16

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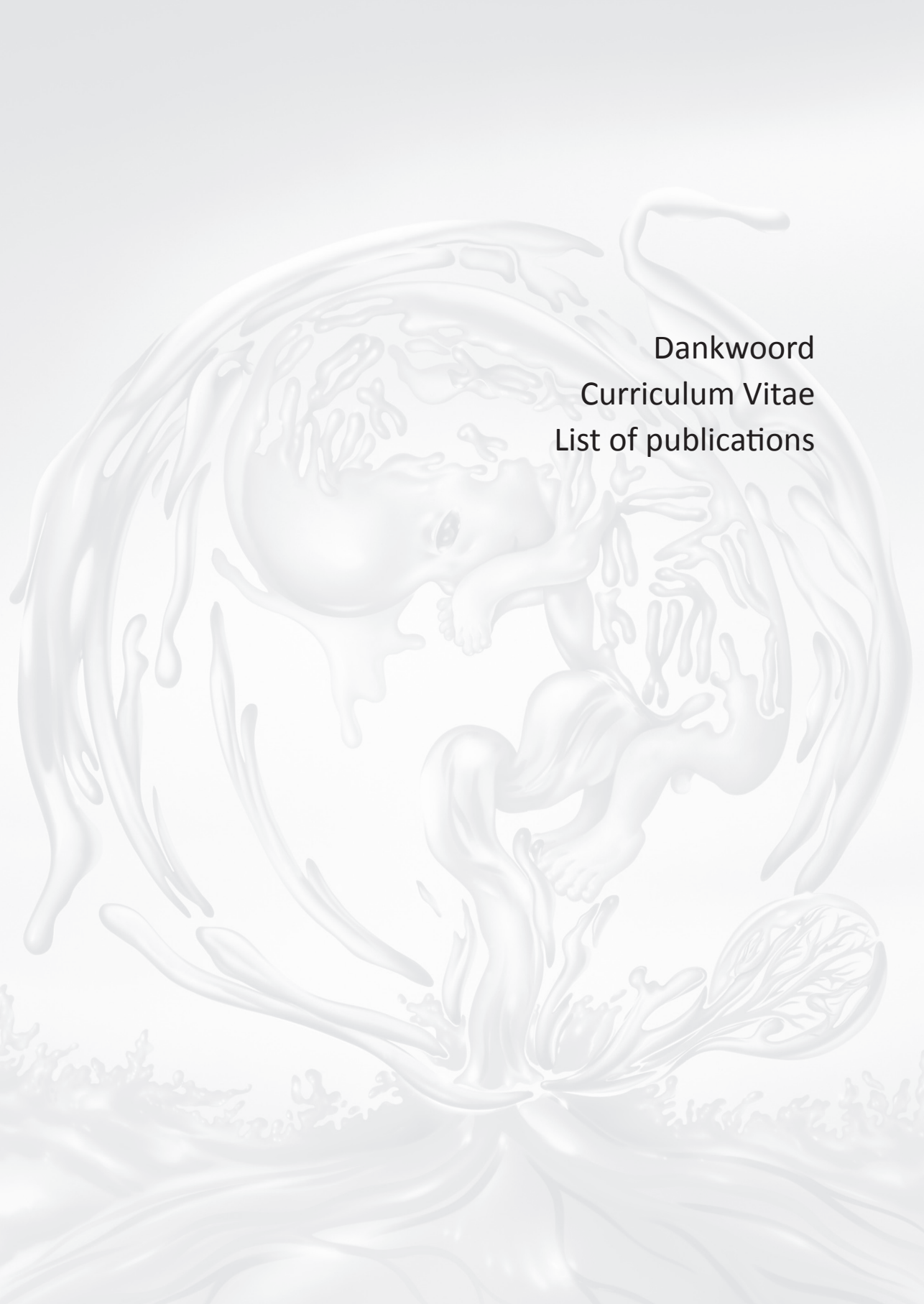

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Dankwoord

2010, een fantastisch jaar voor de Nederlandse sport. Het jaar waarin de Giro d'Italia en de Tour de France op Nederlandse bodem van start gingen. Het jaar waarin vier gouden medailles werden behaald tijdens de Olympische Winterspelen in Vancouver. En natuurlijk het jaar waarin Oranje eindelijk weer eens de finale van het WK haalde, maar helaas net naast de wereldtitel greep.
Promoveren is als topsport; het overwinningsgevoel als een experiment gelukt is, de teleurstelling als een artikel niet geaccepteerd wordt en de frustratie als de data-analyse steeds weer opnieuw moet. Het vergt een flinke dosis doorzettingsvermogen, maar uiteindelijk word je beloond met het eindresultaat: het proefschrift.
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Curriculum Vitae

Wendy Koster was born in Gouda, the Netherlands on August 16th, 1983. She attended secondary school at the 'Coenecoop College' in Waddinxveen, from which she graduated in 2001. That same year she started her medical study at Utrecht University. In 2006, after successfully completing her internships, she conducted a six-month research project on the quality of current Down syndrome screening at the Dutch National Institute for Public Health and the Environment (RIVM) under supervision of Dr. P.C.J.I. Schielen, Dr. Ph. Stoutenbeek and Prof. Dr. G.H.A. Visser. Subsequently, she performed her final internship at the department of Obstetrics and Gynaecology of the Meander Medical Centre in Amersfoort. After graduating as a medical doctor in October 2007, she continued her research project at the RIVM as a PhD-student, which led to the publication of this thesis. For her research she was awarded the Young Investigator Award of 2009, put up by the Centre of Infectious Disease Control of the RIVM.

During her PhD-project she became a member of the International Prenatal Screening Group (IPSG) and an editorial member of the Dutch Association for Community Genetics and Public Health Genomics (NACGG). For a short period of time she worked at the Leeds Screening Centre, UK, where she got acquainted with prediction models for prenatal screening, under supervision of Prof. Dr. H.S. Cuckle. In 2008 she started a master in Clinical Epidemiology at the Utrecht University Graduate school of Life Sciences from which she will graduate in the summer of 2010.

List of publications

1. E.J. Wortelboer, **M.P.H. Koster**, Ph. Stoutenbeek, J.G. Loeber, G.H.A. Visser, P.C.J.I. Schielen. *Fifteen years of triple tests in the Netherlands; the life cycle of a screening test.* Prenat Diagn. 2008 Sep 29;28(10):950-955

2. **M.P.H. Koster**, M. van Leeuwen-Spruijt, E.J. Wortelboer, Ph. Stoutenbeek, L.H. Elvers, J.G. Loeber, G.H.A. Visser, P.C.J.I. Schielen. *Lack of standardisation in determining gestational age for prenatal screening.* Ultrasound Obstet Gynecol. 2008 Oct;32(5):607-11

3. P.C.J.I. Schielen, **M.P.H. Koster**, L.H. Elvers, J.G. Loeber. D*ownsyndroom kansbepaling met de eerste trimester combinatietest 2004-2006 (deels 2007).* RIVM report 230024002/2008 (Dutch)

4. E.J. Wortelboer, **M.P.H. Koster**, Ph. Stoutenbeek, P.C.J.I. Schielen, G.H.A. Visser. *Biochemische screening op pre-eclampsie in het eerste trimester.* In: Reproductieve geneeskunde, gynaecologie en obstetrie anno 2009, E. Slager (ed.). Haarlem: DCGH medische uitgeverij, 2009. ISBN 978-90-811646-5-8. p251-255 (Dutch)

5. **M.P.H. Koster**, E.J. Wortelboer, M.A.J. Engels, Ph. Stoutenbeek, L.H. Elvers, G.H.A. Visser, P.C.J.I. Schielen. *Quality of nuchal translucency in the Netherlands; a quantitative analysis.* Ultrasound Obstet Gynecol. 2009 Jun 26;34(2):136-141

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7. **M.P.H. Koster**, J.L.A. Pennings, S. Imholz, W. Rodenburg, G.H.A. Visser, A. de Vries, P.C.J.I. Schielen. *Bead-based multiplexed immunoassays to identify new biomarkers in maternal serum to improve first trimester Down syndrome screening.* Prenat Diagn. 2009 Sep;29(9):857-62

8. E.J. Wortelboer, I.H. Linskens, **M.P.H. Koster**, Ph. Stoutenbeek, H. Cuckle, M.A. Blankenstein, G.H.A. Visser, J.M.G. van Vugt, P.C.J.I. Schielen. *ADAM12s as a first-trimester screening marker of trisomy.* Prenat Diagn. 2009 Sep;29(9):866-9

9. **M.P.H. Koster**. *Detection for false-positive rate – Depends on age distribution.* PS Perspectives 2009 Vol 15, p24

10. J.L.A. Pennings, **M.P.H. Koster**, W.Rodenburg, P.C.J.I. Schielen, A. de Vries. *Discovery of novel serum biomarkers for prenatal Down Syndrome screening by integrative data mining.* PLoS ONE 2009 Nov 24;4(11):e8010

11. **M.P.H. Koster**, E.J. Wortelboer, H.S. Cuckle, Ph. Stoutenbeek, G.H.A. Visser, P.C.J.I. Schielen. *Placental protein 13 as a first trimester screening marker for aneuploidy.* Prenat Diagn. 2009 Dec;29(13):1237-41

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15. **M.P.H. Koster**, P.C.J.I. Schielen, L.H. Elvers, J.G. Loeber. D*ownsyndroom kansbepaling met de eerste trimester combinatietest 2006-2008.* RIVM report 230083001/2010 (Dutch)

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17. E.J. Wortelboer, **M.P.H. Koster**, H.S. Cuckle, Ph. Stoutenbeek, G.H.A. Visser, P.C.J.I. Schielen. *First-trimester PP13 and PlGF: reliable markers for the detection of early preeclampsia.* BJOG. [accepted for publication]

18. **M.P.H. Koster**, J.L.A. Pennings, S. Imholz, W. Rodenburg, G.H.A. Visser, A. de Vries, P.C.J.I. Schielen. *Proteomics and Down syndrome screening: Mixed blessings from a validation study.* [in revision]

19. **M.P.H. Koster**, E.J. Wortelboer, Ph. Stoutenbeek, G.H.A. Visser, P.C.J.I. Schielen. *Modeling the Down syndrome screening performance using first trimester serum markers*. [submitted]

20. S. Kuc, E.J. Wortelboer, **M.P.H. Koster**, H.W. de Valk, P.C.J.I. Schielen, G.H.A. Visser. *Biomarkers of early placental function in IDDM pregnancies; relationship to fetal growth.* [submitted]