Prenatal screening for aneuploidies and adverse pregnancy outcome: the significance of second trimester soft markers and low first trimester PAPP-A

Marja Kaijomaa

Academic dissertation

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To my family
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ABBREVIATIONS

AC  Amniocentesis
AFP  Alpha-fetoprotein
ARSA  Absent right subclavian artery
BP  Blood pressure
BPD  Biparietal diameter
cffDNA  cell free fetal DNA
CI  Confidence interval
CHD  Congenital heart disease
CNS  Central nervous system
CPC  Choroid plexus cyst
CRL  Crown-rump length
CS  Caesarean section
CVS  Chorionic villus sample
DNA  deoxyribonucleic acid
DR  Detection rate
DS  Down syndrome
EB  Echogenic bowel
EIF  Echogenic intracardial focus
EFW  Estimated fetal weight
FGR  Fetal growth restriction
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>FL</td>
<td>Femur length</td>
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<td>FMU</td>
<td>Fetal Medicine Unit</td>
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<td>FPR</td>
<td>False positive rate</td>
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<td>FTS</td>
<td>First trimester screening</td>
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<td>gw</td>
<td>Gestational weeks</td>
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<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
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<tr>
<td>HUH</td>
<td>Helsinki University Hospital</td>
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<tr>
<td>LR</td>
<td>Likelihood ratio</td>
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<tr>
<td>MC</td>
<td>Miscarriage</td>
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<tr>
<td>MoM</td>
<td>Multiple of Median</td>
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<tr>
<td>MVM</td>
<td>Mild ventriculomegaly</td>
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<tr>
<td>NB</td>
<td>Nasal bone</td>
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<td>NF</td>
<td>Nuchal fold</td>
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<tr>
<td>NIPT</td>
<td>Non-invasive prenatal test</td>
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<tr>
<td>NT</td>
<td>Nuchal translucency</td>
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<tr>
<td>NTD</td>
<td>Neural tube defect</td>
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<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>PAPP-A</td>
<td>Pregnancy-associated plasma protein A</td>
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<tr>
<td>PE</td>
<td>Pre-eclampsia</td>
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<td>PI</td>
<td>Pulsatibility Index</td>
</tr>
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<td>PIH</td>
<td>Pregnancy induced hypertension</td>
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<tr>
<td>PPV</td>
<td>Positive predictive value</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PTD</td>
<td>Preterm delivery</td>
</tr>
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<td>PY</td>
<td>Pyelectasis</td>
</tr>
<tr>
<td>SA</td>
<td>Spontaneous abortion</td>
</tr>
<tr>
<td>SB</td>
<td>Stillbirth</td>
</tr>
<tr>
<td>SF</td>
<td>Short fetal femur</td>
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<tr>
<td>SFLB</td>
<td>Short fetal long bones</td>
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<tr>
<td>SGA</td>
<td>Small for gestational age</td>
</tr>
<tr>
<td>SH</td>
<td>Short fetal humerus</td>
</tr>
<tr>
<td>uE3</td>
<td>unconjugated estriol</td>
</tr>
<tr>
<td>UmaD</td>
<td>Umbilical artery Doppler</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound</td>
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<tr>
<td>UtaD</td>
<td>Uterine artery Doppler</td>
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<tr>
<td>TOP</td>
<td>Termination of pregnancy</td>
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ABSTRACT

**Background**: Soft sonographic markers were introduced into screening for aneuploidies in the 1980s. The main purpose was to increase the detection of Down syndrome (DS), since these minor anatomic markers were more often detected in fetuses with DS. The use of second trimester soft markers in screening for aneuploidies has continued, even though the first trimester nuchal translucency (NT) measurement alone and combined with the assessment of maternal serum markers became the standard of DS screening in the 1990s. Subsequently, the value of second trimester soft markers in screening for DS has been questioned. However, some soft markers still have significance as markers of adverse outcomes.

Today, the primary screening method for DS is the combined first trimester screening (FTS). It is an effective screening method and constitutes the measurement of fetal NT together with the assessment of maternal serum pregnancy-associated plasma protein A (PAPP-A) and free beta subunit of human chorionic gonadotropin (hCG). Since PAPP-A has the ability to influence mitogenic activity, differentiation and placentation, it has also been widely studied as a potential marker of adverse pregnancy outcome.

**Aims**: The aim of the study was to assess the significance of soft markers in screening for DS after normal FTS, and to evaluate the significance of isolated second trimester short fetal femur (SF) and short fetal humerus (SH) in euploid pregnancies. Another aim of was to estimate the outcome of pregnancies with low first trimester PAPP-A and to assess the association of pregnancy outcome with the level of PAPP-A.

**Subject and methods**: This dissertation consists of two separate study lines. First, 228 singleton pregnancies with a normal FTS and two or more soft markers in the second trimester ultrasound (US) were studied. The number of aneuploidies was retrospectively analyzed. In addition, 30 euploid pregnancies with isolated SF and SH in the second trimester US were studied and the incidence of adverse outcomes was analyzed.

Second, 961 pregnancies with low (<0.3 MoM) first trimester PAPP-A were studied. The incidence of adverse outcome was studied and compared to 961 pregnancies with optimal
PAPP-A levels, i.e. 0.9-1.1 multiples of median (MoM). Pregnancies with low PAPP-A were further divided into three subgroups (PAPP-A levels <0.1 MoM, 0.1-0.2 MoM and 0.2-0.3 MoM) and the association of adverse outcome with PAPP-A levels was analyzed.

**Results**: After normal FTS, only two significant aneuploidies (0.9%) were detected. They included one case of trisomy 18 and a case of triploidy. In addition, a case of 4% trisomy 21 mosaicism and a case of chromosome 10 inversion were detected. These pregnancies ended up in the delivery of an apparently healthy newborn. Euploid pregnancies with a SF and SH in the second trimester US had a significantly increased risk of preterm delivery (PTD), pre-eclampsia (PE), a small for gestational age (SGA) newborn, an emergency Caesarean delivery, stillbirth (SB) and perinatal death.

Compared to pregnancies with optimal PAPP-A levels, pregnancies with low PAPP-A had an increased risk of aneuploidy and spontaneous abortion (SA), but not of structural anomaly. Also, the risk of PTD, PE, an SGA newborn and SB was significantly increased, and the risk of emergency Cesarean delivery was slightly increased. The risk of aneuploidy, SA, PTD and an SGA newborn was associated with the level of low PAPP-A and increased as levels of PAPP-A decreased.

**Conclusions**: After normal FTS, second trimester soft markers have low specificity for detecting DS, but a chromosomal abnormality was found in 0.9% of pregnancies. The risk of adverse pregnancy outcomes is significantly increased in euploid pregnancies with SF and SH. Also, the risk of adverse pregnancy outcomes is increased in pregnancies with low first trimester PAPP-A levels and the risk is often inversely associated with the level of PAPP-A.
INTRODUCTION

Over the last four decades, prenatal screening has been used to detect fetal abnormalities during pregnancy. It started in the mid-1970s in order to identify pregnancies affected with neural tube defects (NTD) by measuring maternal serum alpha-fetoprotein level (AFP) during the second trimester of pregnancy.

Since the 1980s, the focus of prenatal screening has been on the detection of pregnancies with Down syndrome (DS). At first, it included the measurement of second trimester maternal serum markers associated with an increased risk of DS. Also, an enlarged fluid-filled nuchal subcutaneous space, a nuchal fold (NF), was detected by ultrasound (US) and it was found to be associated with DS (Benacerraf and Frigoletto 1987). Subsequently, advances in US technology enabled the detection of even smaller structural details and some minor variations of anatomy were found to be more common in fetuses with DS. Thereafter, these so-called soft markers were also used to identify fetuses with DS.

Later on, the enlarged nuchal space of DS fetuses was described earlier in pregnancy (Nicolaides et al. 1992, Ville et al. 1992), and the US screening was shifted to the first trimester. At the first trimester, this fluid-filled nuchal space was called nuchal translucency (NT). To increase the detection rate (DR) of DS, the evaluation of NT was later combined with the measurement of maternal serum parameters. Together they form the combined first trimester screening (FTS), the primary screening method used today.

When an increased risk of DS is detected in prenatal screening, counseling and further diagnostic tests are offered to test the fetal karyotype. Traditional methods, the chorionic villus sample (CVS) and amniocentesis (AC), carry a small risk of miscarriage (MC) as a procedure-related complication. In recent years however, the possibility of a non-invasive prenatal test (NIPT) has been available, offering a more sensitive method of screening without any risks.
Prenatal screening is a common practice in developed countries and is offered to all pregnant women in Finland. Due to many technological advancements, most aneuploidies can be detected during pregnancy. Ongoing research will continue to evolve prenatal screening in the future and the availability of increased prenatal information will raise new ethical dilemmas.

Preterm delivery (PTD), pre-eclampsia (PE), stillbirth and poor fetal growth still remain the challenges of prenatal care. Since these adverse maternal and fetal outcomes of pregnancy are much more common than NTDs and DS, many attempts have been made to develop screening for unfavorable outcomes of pregnancy. Since many adverse outcomes originate from placental events in the first trimester, attempts have been made to use maternal first and second trimester placenta-derived serum parameters as outcome markers. Their association with pregnancy outcome has been studied for decades. One marker of enormous interest has been the pregnancy-associated plasma protein A (PAPP-A) which, along with being associated with aneuploidy, has been shown to be associated with adverse outcomes like PTD, spontaneous abortion (SA), pre-eclampsia PE and poor fetal growth. Even though the risk of adverse pregnancy outcome seems to increase with lower levels of PAPP-A, the positive predictive value (PPV) remains low. Thus, the opinions concerning the use of PAPP-A as a screening tool are controversial.

The respect for patient autonomy is the main principle of all prenatal screening. It is achieved with sufficient pre-screening information, which enables an informed choice concerning the decision of participation in prenatal screening, further diagnostic tests and the decision concerning the future of the affected pregnancy.

The aim of this study was to evaluate the clinical significance of soft markers as markers for DS in the population already screened for DS in the first trimester. Another aim was to evaluate the significance of short fetal long bones (SFLB) in fetuses not affected by aneuploidy. Since the significance of low PAPP-A levels in the literature is disputable, we also wanted to evaluate outcomes of these pregnancies in our screening population.
REVIEW OF THE LITERATURE

Screening

Screening in general means a process of identifying individuals from an apparently healthy population at an increased risk for a condition or disease. It is not the same as diagnosis, but it divides the screened population into those at high risk (i.e., screen positive) and low risk (i.e., screen negative). Counseling, further testing and appropriate treatment is offered to reduce the risk or complication arising from the disease or condition screened for.

Screening tests must be sensitive enough to find the population with the condition screened for, referred to as true positives. It should also be specific enough not to select individuals without the screened condition, the true negatives. The sensitivity and specificity of the screening test are presented as percentages of the screened population. The PPV signifies the likelihood (%) of the screened condition to be truly present in the screen positive population.

Most people with a screen positive result are healthy and the condition is not found upon further examinations. This population is referred to as false positives. Also, some people will pass the screening despite being affected with the screened condition and are referred to as false negatives. When the sensitivity of the screening test increases, the screened condition will be more accurately detected. Improving sensitivity, however, occurs at the expense of specificity, thereby increasing the false positive rate (FPR).

Screening should always be voluntary. Sufficient information has to be available for individuals attending the screening to understand the possibilities and limitations of screening.

The principles of screening from the World Health Organization (Wilson and Jungner 1968) and the Danish Council of ethics (The Danish Council of ethics 2000) are as follows:
- The condition is important to the individual and society
- The prevalence of the condition is sufficiently high
- The condition can be screened before its natural appearance
- The condition is severe and its natural course is well known
- A cost-effective and acceptable screening tool is available
- The effectiveness of the screening is evaluated; the specificity and sensitivity of the screening test are sufficiently high
- Facilities for diagnosis are available and they are generally accepted
- An acceptable treatment and a policy on who to treat exists
- Social and ethical aspects of screening are evaluated
- Screening is continuous

The purpose of prenatal screening

The main purpose of prenatal screening is to assess whether the pregnant women has an increased risk of having a fetus with chromosomal or structural abnormalities. Chromosomal abnormalities are detected in approximately 0.5% of newborns and are common enough to be screened. However, no treatment is available. Major structural abnormalities are detected in 2-3% of newborns (Autti-Rämö et al. 2005), which makes the screening for structural abnormalities justified.

During the years 1993-2002, the incidence of the most common chromosomal abnormality, DS, in Finland was 12.2-14.2/10,000 newborns; 5% were stillborn and 6% died during infancy. The incidence of structural abnormalities among newborns was 2.9%. When including terminations of pregnancy (TOP) due to abnormality, the incidence was approximately 3.3%. The most common abnormalities were structural defects of the heart, gastrointestinal tract and central nervous system (CNS). More than 200 TOPs per year were performed due to fetal structural abnormalities, and 5% of newborns with structural abnormality died, despite treatment (Autti-Rämö et al. 2005).

The risk of chromosomal abnormalities increases with maternal age, but age does not significantly affect the risk of structural abnormalities. Gastoscisis is an exception, with a higher risk among young mothers (Autti-Rämö et al. 2005). The risk of DS as a function of maternal age is shown in Figure 1.
Most common chromosomal abnormalities

Human cells normally contain 23 pairs of chromosomes, for a total number of 46 chromosomes. Of these, 22 pairs are autosomes and look similar in males and females. In the 23rd pair, the sex chromosomes, women have two X chromosomes while men have one X and one Y chromosome.

During fertilization, a set of 23 chromosomes from both parents are combined to the normal number of 46 chromosomes. This is called a euploidic genome. An uneven separation of chromosomes during cell division prior to fertilization results in extra or missing chromosomes. The presence of an abnormal number of chromosomes is called aneuploidy. Aneuploidies are a common cause of genetic disorders and birth defects and are a risk factor for SA and fetal death (Practice Bulletin No. 163 Summary: Screening for Fetal Aneuploidy. 2016).

The most common chromosomal abnormality is trisomy 21, also known as DS (Chou et al. 2009, Roizen and Patterson 2003). The risk of DS increases with increasing maternal age.

Figure 1. Maternal age and the number of DS cases (years 1993-2002)
(Chou et al. 2009, Muggli and Halliday 2004, Roizen and Patterson 2003). Due to associated anomalies, the risk for SA, stillbirth (SB) and infant death is increased in pregnancies with DS. In a large European study, 43.6% of newborns with DS had a congenital heart disease (CHD) and 15% had a non-cardiac anomaly (Morris et al. 2014). The risk of hearing loss (Austeng et al. 2013, Manickam et al. 2016) and leukemia (Izraeli 2016) is also substantially increased. Additionally, DS is the most common cause of mental retardation (Rachidi and Lopes 2007), and 75% of DS individuals develop symptoms of Alzheimer’s disease (Roizen and Patterson 2003). However, many liveborn DS infants also survive into adulthood in good health. In Finland, approximately 60-70 newborns with DS are born every year (Autti-Rämö et al. 2005).

After DS, trisomies 18 and 13 are the most common autosomal trisomies. Trisomy 18, also called Edwards syndrome, is usually associated with severe growth restriction and multiple malformations; most typically major cardiac abnormalities, NTDs, renal abnormalities and club or rocker-bottom feet. Edwards syndrome fetuses also tend to have a thickened NF, a strawberry-shaped skull and cisterna magna abnormalities. About 80-86% of fetuses with trisomy 18 are detected prenatally. In a study by Cavadino and Morris (2017), 30% of viable fetuses at 12 weeks of pregnancy resulted in a live birth. In the absence of prenatal screening and selective TOP, the live birth prevalence is estimated to be 2.3/10 000 (Savva et al. 2010).

Trisomy 13 (Patau syndrome), is associated with multiple malformations, such as CHD, CNS anomalies and NTDs, and has a sonographic DR of 90-100% (Shipp and Benacerraf 2002). Approximately 50% of viable pregnancies at 12 weeks of pregnancy result in a live birth (Cavadino and Morris, 2017). The estimated prevalence in the absence of prenatal screening and TOP is 1.4/10 000 births (Savva et al. 2010). Trisomies 18 and 13 have both a high risk of SB, with only 1 as 12 surviving for one year or more. The median postnatal survival is two weeks (Wu et al. 2013).

Triploidic fetuses have an excessive complete set of chromosomes from either parent. Fetuses typically have multiple congenital abnormalities, most commonly brain, cardiac or renal abnormalities, NTDs and club foot. Asymmetric growth restriction, oligohydramnion and a small placenta are typical features when the excessive set of chromosomes is maternal. A hydropic placenta with partial mole is typical when excessive chromosomes are paternal. The
majority of triploidic pregnancies are lost in early gestation (Jauniaux et al. 1997, Zalel et al. 2016).

Abnormalities of sex chromosomes are slightly less common than autosomal chromosome abnormalities. They are less severe in their effects and rarely lethal. The most common sex chromosome abnormality in females is Turner syndrome (45X). These pregnancies most often end in MC (Papp et al. 2006) and the prevalence among liveborns is approximately 3/10,000 (Gravholt et al. 1996). Fetuses with Turner syndrome often present with a very thick NT (Pinsker 2012) and intrauterine growth restriction (IUGR) in later pregnancy (Papp et al. 2006). If the pregnancy survives to birth, short stature, a thick neck, premature ovarian failure and aortic coarctation are typical features (Gruchy et al. 2014, Papp et al. 2006, Saenger et al. 2001).

The most typical sex chromosome abnormality in men is Klinefelter Syndrome (47XXY) and the prevalence among liveborn infants is approximately 17/10,000 (Morris et al. 2008). Most individuals develop as males without knowing they possess an extra X chromosome. Some will develop varied or subtle characteristics of the syndrome which include reduced fertility, gynecomastia, small testicles and tall stature (Giltay and Maiburg 2010).

**Screening in Finland**

In Finland, prenatal screening is offered to all pregnant women registered at the antenatal clinics. It is voluntary and free of charge.

During the pre-screening appointments, both oral and written information are given concerning the screening options for chromosomal and structural abnormalities. An informed consent concerning the participation or refusal should be given by the pregnant woman. The vast majority of pregnant women opt to participate in prenatal screening.

Finnish national prenatal screening programs were harmonized in the beginning of 2010. Screening is regulated by the Governmental Decree on Screening (Health Care Act 339/2011) and organized by local authorities as part of the general healthcare. The present screening
program includes the general US examination during early pregnancy, screening for aneuploidies in the first trimester and for structural abnormalities during the second trimester.

For social indications, a TOP is allowed up to $12^{+0}$ gestational weeks (gw) (Laki raskauden keskeyttämisestä 24.3.1970/239, ‘The Finnish abortion law’). It requires the examination and consideration of two authorized physicians. TOP for fetal indications requires the permission from the national Abortion Committee in the National Supervisory Authority of Welfare and Health (Valvira). Between $12^{+1}$-$20^{+0}$ gw, TOP is allowed in cases with exceptional social indications and in cases of a suspected or diagnosed severe fetal developmental defect, illness or handicap. In cases with a diagnosis of severe fetal abnormality, TOP is allowed up to $24^{+0}$ gw. In Finland, a TOP for fetal indications is illegal after $24^{+0}$ gw. Available options in prenatal screening are presented in Figure 2.
US=ultrasound, TOP=termination of pregnancy, NT=nuchal translucency, P=abnormal
First trimester screening

The general US at 10\textsuperscript{0}-13\textsuperscript{+6} gw is offered to all pregnant women. The indication for the exam is the verification of viability and location of the pregnancy, number of fetuses and the gestational age. The screening for fetal chromosomal and structural abnormalities is combined with the general US based on informed consent of the pregnant woman.

Screening for chromosomal abnormalities

Since the risk of DS increases with increasing maternal age (Kurtovic-Kozaric et al. 2016, Savva et al. 2010), the screening of chromosomal abnormalities began by offering karyotyping to older pregnant women. It was estimated that when offering karyotyping to women over 37 years of age, the DR of DS would be approximately 30%, with an invasive test rate of 5% (Snijders et al. 1998).

Nowadays, screening for chromosomal abnormalities is based on the first trimester general US. It is combined with the measurement of NT and, if opted for, with a measurement of maternal serum markers of DS.

Ultrasound markers for chromosomal abnormalities

In the 1980s, an enlarged fluid-filled fetal nuchal subcutaneous space was associated with DS. It was first described in the second trimester and referred to as a NF (Benacerraf and Frigoletto 1987). Later, in 1992, this enlarged fluid-filled subcutaneous nuchal space was detected in fetuses with DS during the first trimester. At this stage, it was referred to as NT (Nicolaides et al. 1992, Ville et al. 1992). Since then, the measurement of NT at the first trimester general US screening has been offered to pregnant women opting for chromosomal screening.
The risk of DS increases with increasing NT. In the risk assessment, the fetal gestational age has to be taken into account, since NT also increases with increasing crown–rump length (CRL) (Braithwaite et al. 1996). If combining the NT information with the risk based on maternal age, the DR of DS increases to 75%, with a 5% FPR (Snijders et al. 1998).

The absence or hypoplasia of a fetal nasal bone (NB) is a strong indicator of chromosomal abnormality (Odibo et al. 2008, Sonek et al. 2006). The NB is absent in approximately 60% of fetuses with DS, compared to 2.6% of euploid fetuses (Bromley et al. 2002a). The NB can be visible at a CRL length of 42 mm, corresponding to 11 gw (Sandikcioglu et al. 1994), and in euploid fetuses, the bone length increases as a function of gw (Sonek et al. 2003). NB hypoplasia has been defined by using a fixed cut-off of 2.5 mm or 3.0 mm (Cicero et al. 2003), by gestation-specific percentiles (Bunduki et al. 2003), multiples of medians (MoM) (Gianferrari et al. 2007, Odibo et al. 2007) and by biparietal diameter (BPD)-to-NB length ratio (Bromley et al. 2002a, Tran et al. 2005a). It is worth noting that ethnicity effects the NB length (Casasbuenas et al. 2009, Chen et al. 2006, Cossi et al. 2008, Ozer et al. 2010), with the absence of NB being more common in Afro-Caribbean euploid fetuses compared to white fetuses (Gautier et al. 2016). Recently, the NB evaluation has been brought into screening in many centers and the NB length evaluation (MoM) with ethnic and gestational age corrections is widely accepted as part of FTS (Odibo et al. 2007, Sonek 2007, Wapner 2008).

A reversed a-wave in the ductal vein and tricuspid valve regurgitation are also markers associated with DS. They are present in 65% and 55% of fetuses with DS compared to only 3.2% and 0.9% of euploid fetuses, respectively (Stressig et al. 2011). Regardless of their strong association with DS, apart from NT, the US markers presented here are not used as part of regular first trimester US screening at Helsinki University Hospital (HUH).

Combined FTS screening for chromosomal abnormalities

Today, the primary first trimester chromosomal screening method is the combined FTS. It includes the first trimester US parameters, together with specific maternal serum markers. The general US screening with NT measurement is performed at 11⁺⁰-13⁺⁶ gw, corresponding to a
fetal CRL between 45 and 84 mm, and the serum test is performed at 9<sup>th</sup>-11<sup>th</sup> gw to measure maternal serum levels of PAPP-A and free beta-hCG. The best accuracy is achieved when the serum test is taken at 9-10 gw (Kirkegaard et al. 2008, Wright et al. 2010), but it can also be taken at the same time as the NT measurement (OSCAR, i.e. one-stop clinic for assessment of risk) (Kagan 2008). The former strategy has been used at HUH since the beginning of 2013 and a cut-off value of 1/250 is used to determine an increased risk. Combined FTS is only used in singleton pregnancies.

The risk for DS is calculated based on the serum markers, NT and maternal age. The serum markers are corrected for maternal weight, smoking and diabetes. This method has been reported to achieve a DR of 63-90% with an FPR of 3-5% (Ekelund et al. 2015, Kagan 2008, Kagan et al. 2009, Malone et al. 2005, Nicolaides 2005, Ranta et al. 2011a). Currently, the combined FTS is also used to evaluate the risk for trisomy 18 (Huttly and Wald 2011). At HUH, this was introduced in the beginning of 2012 and a cut-off value of 1/100 is used to determine an increased risk.

**Screening for adverse pregnancy outcome**

**Screening for structural abnormalities**

The primary purpose of FTS is not to look for fetal structural abnormalities. However, severe abnormalities in the CNS, gastrointestinal system, urinary tract or limbs may already be visible at the FTS.

Increased NT is associated with an increased risk of structural abnormalities (Goldstein et al. 2014, Souka et al. 2001, Weiner et al. 2007, Williams 2005). CHDs comprise the majority of defects, but diaphragmatic hernia, pulmonary, genitourinary, musculoskeletal and mid-line defects are also reported (Baer et al. 2014, Souka et al. 2001, Timmerman et al. 2010).
Screening for other adverse pregnancy outcomes

Numerous studies have investigated techniques and biomarkers with the aim of already predicting adverse pregnancy outcomes during the first trimester (Halscott et al. 2014). The prediction of PE, PTD, gestational diabetes, SB and poor fetal growth have been studied. The early identification of risks would be beneficial for counseling, streamlining pregnancy follow-up and interventions (D’Antonio et al. 2013, Sharp and Alfrevic 2014).

Maternal serum screen parameters of the combined FTS have been widely used in the attempt to screen for adverse pregnancy outcomes during the first trimester of pregnancy. The research is based on the assumption that adverse outcomes are associated with placental insufficiency, which could be diagnosed early by evaluating the levels of maternal serum parameters of the combined FTS.

PAPP-A is studied as an independent marker of adverse pregnancy outcomes. It is a proteinase produced by the syncytiotrophoblasts of the placenta and is responsible for the cleavage of insulin-like growth factor binding protein-4 from insulin-like growth factor. PAPP-A is inherently linked to the function of the placenta and recent evidence also suggests an association of insulin-like growth factor binding protein-4 and PAPP-A with the regulation of bone development (Qin et al. 2006) and skeletal growth and remodeling (Conover et al. 2004, Miyakoshi et al. 2001, Ortiz et al. 2003).


However, the PPV for most adverse outcomes is poor. According to several studies, the PPV for IUGR and SGA was approximately 2.97 (Carbone et al. 2012, Marttala et al. 2010, Peterson and Simhan 2008, Spencer et al. 2008b), and no association of PAPP-A <10th and
<5th centile and poor fetal growth was found in some studies (Saruhan et al. 2012, van Ravenswaaij et al. 2011). Also, the evidence regarding low PAPP-A and hypertensive disorders in pregnancy is mixed (Odibo et al. 2011, Poon et al. 2009a, Saruhan et al. 2012, van Ravenswaaij et al. 2011).

The association of low PAPP-A with PTD in previous studies is conflicting (Canini et al. 2008, Goetzinger et al. 2010, Kirkegaard et al. 2010, Saruhan et al. 2012, van Ravenswaaij et al. 2011), as well as the association with SB (Akolekar et al. 2011, Marttala et al. 2010, van Ravenswaaij et al. 2011, Proctor et al. 2009). Studies concerning the risk of MC are more in agreement, with low PAPP-A levels seeming to increase the risk of MC (Hanita et al. 2012, van Ravenswaaij et al. 2011, Yaron et al. 2002a).

Conversely, high PAPP-A levels (> 90th centile) have been associated with a risk of large for gestational age fetuses, with an adjusted OR of 2.9. (Canini et al. 2008). Different cut-off levels for low and high PAPP-A and altering determinations of adverse outcomes significantly hamper the comparison of previous studies.

Low levels of hCG in the first trimester of pregnancy have been associated with an increased risk of birthweight < 5th or 10th centile (Canini et al. 2008, Dugoff et al. 2004), although all authors do not agree with this (Canini et al. 2008). Low levels, as well as high levels (Heinonen et al. 1996), of hCG have been shown to be associated with hypertensive disorders (Canini et al. 2008), with extremely low levels (<0.25 MoM) being associated with an increased risk of MC before 24 weeks (Dugoff et al. 2004, Rissanen et al 2006). Cut-off levels and outcome determinations also vary among the studies concerning hCG, therefore, the screening capacity of both PAPP-A and hCG in the first trimester is low.

Poor invasion of the extravillous placental trophoblasts to the endometrium and maternal spiral arteries results in retention of an abnormal vasoconstrictive capacity in the maternal spiral arteries (Smith et al. 2009). This can be identified with Doppler US within the maternal uterine arteries that are located upstream from the spiral arteries. In previous studies, the first trimester uterine artery Doppler (UtaD) was able to predict early-onset PE or PE with sensitivities of 47.8% and 26.4% with PPVs of 7.9% and 6.6%, respectively (Spencer et al. 2008, Velauthar et al. 2014). When used in combination with maternal factors, the DR has been shown to
increase to 81% with a 10% FPR. However, due to high FPR and low sensitivity, the first trimester UtaD should not be used in screening for poor fetal growth (Barrett et al. 2008, Velauthar et al. 2014).

**Second trimester screening**

The DS serum screening was originally designed and started in the second trimester. In the beginning, it included only the evaluation of maternal serum AFP and hCG. To produce a better DR, unconjugated estriol (uE3) was later added to form the triple test, and finally a fourth marker, inhibin-A, was added to form a quadruple test of the second trimester. These tests include the risk assessment based on maternal age and serum markers.

**Screening for chromosomal abnormalities**

It is also possible to perform the serum screening for chromosomal abnormalities in the second trimester. Compared to combined FTS, the DR for DS is lower, but the test is feasible, for example, in situations of late registration to the antenatal clinic or incorrect dating of the pregnancy in the first trimester US. Soft US markers for DS have been used since the 1980s and are used as the second part of the chromosomal screening in the second trimester.

**Serum screening for chromosomal abnormalities**

In Finland, the serum screening for chromosomal abnormalities can be performed optionally during the second trimester at 15\(^{+10}-16^{+6}\) gw. The second trimester serum screening in HUH includes the measurement of maternal AFP and hCG. The risk cut-off 1/250 is used for further examinations. In Finland, approximately 600 mothers per year attend the second trimester serum screening (personal communication: Jarkko Rompanen, ISLAB). In Finland, first and second trimester serum screening options cannot both be chosen and serum screening can only be made in singleton pregnancies.
Ultrasound markers for chromosomal abnormalities

Major markers of aneuploidy

DS, as well as other aneuploidies, are associated with an increased risk for structural anomalies, most often in the heart, gastrointestinal or genitourinary tract and in the CNS (Hansmann 2004, Nicolaides 2003, Stoll et al. 2015). In previous studies, major abnormalities were detected in 21.4-28.5% of fetuses with DS (Nicolaides 2003, Papp et al. 2006).

Heart defects are common in pregnancies with aneuploidy and are estimated to be present in 16-44% of pregnancies with DS (Hansmann 2004, Papp et al. 2007, Stoll et al. 2015). The most typical findings are the atrioventricular and ventricular septal defect. Coarctation of the aorta and tetralogy of Fallon are also relatively frequently seen (Papp et al. 2007, Stoll et al. 2015). The most typical gastrointestinal anomalies in fetuses with DS are duodenal and esophageal atresia (Raniga et al. 2006, Shipp and Benacerraf 2002, Stoll et al. 2015) and hydronephrosis, and obstructive anomalies are described as typical abnormalities in the genitourinary tract (Raniga et al. 2006, Stoll et al. 2015).

Brachycephaly, a shortening of the occipitofrontal diameter of the head, is also commonly seen in fetuses with DS (Hansmann 2004, Shipp and Benacerraf 2002). Another CNS anomaly typically seen in fetuses with DS is ventriculomegaly, a widening of lateral ventricles (Papp et al. 2007, Raniga et al. 2006, Shipp and Benacerraf 2002). However, this marker is often considered as only a minor marker of DS (Signorelli et al. 2004).

Major structural anomalies have a high association with DS and are, for that reason, called major markers of aneuploidy. These anomalies are unlikely to be missed in the second trimester US and fetal karyotyping is advisable when major markers of aneuploidy are detected (Nicolaides 2003).
Minor markers of aneuploidy

Advances in US technology have enabled the detection of even the smallest structural details of the fetus. Since the 1980s, a number of different prenatal sonographic markers have been recognized to be more common in fetuses with DS, and were thereafter studied in an attempt to detect fetuses with DS. These minor variations are also detected in a varying proportion of normal fetuses, but due to their statistical association with DS, they were brought into screening as ‘soft markers’ for DS.

Soft markers are reported to be present in approximately 11-14% of all second trimester US examinations (Van den Hof et al. 2005), and 5.9% (Åhman et al. 2014) and 14.2% (Bromley et al. 2014) in low-risk and previously screened populations, respectively. The use of soft markers is estimated to account for approximately a 4% increase in the detection of aneuploidies (Bethune 2008, Boyd et al. 1998), while it increases the FPR 12-fold. Since many markers were introduced before the use of NT-screening, combined FTS or second trimester serum screening, the applicability of markers has changed after the widespread use of combined FTS.

Nuchal fold

In 1985, an association between a thickening of the fetal nuchal area and DS was reported by Benacerraf and colleagues. This thickening was called the NF and a measurement ≥6 mm was subsequently shown in 40-50% of fetuses with DS, compared to 0.1% of euploid fetuses (Benacerraf and Frigoletto 1987a, Shipp and Benacerraf 2002). The specificity of this marker was estimated to reach 99% (Smith-Bindman et al. 2001), and it was soon accepted as a powerful US marker for DS.

An abnormal NF is considered to be the most powerful marker for DS. It should be used between 15 and 20 weeks of pregnancy (Benacerraf and Frigoletto 1987a, Benacerraf 2002) and the measurement is taken by a modified transverse view of the fetal head. The thickness is measured from the outer edge of the occipital bone to the outer edge of the skin fold (Benacerraf et al. 1992). In the 1990s, Kypros Nicolaides developed a standardized method for nuchal soft tissue measurement in the first trimester, the NT measurement, which has since become the basis of screening for DS. These two measurements are not thought to represent
the same finding, but are rather considered as independent markers for DS (Maymon et al. 2008).

**Short femur and short humerus**

Short stature is a common feature of individuals with DS, and a short fetal femur (SF) and a short fetal humerus (SH) were the next US markers associated with DS (Benacerraf et al. 1987b). The association of SF with an increased risk of DS has been reported by many authors (Agathokleous et al. 2013, Mathiesen et al. 2014, Nyberg and Souter 2001b), although some studies challenge this association (Todros et al. 2004, Vergani et al. 2000). SF is also detected in many fetuses with Turner syndrome (Papp et al. 2006).

SF is determined as a bilateral, symmetric shortening of the bone with normal bone morphology, and the ossified fetal femur diaphysis is measured from the major trochanter to the end of the femoral shaft (Ugurlucan et al. 2012). Definitions for SF in the literature are variable and femur lengths (FL) <5<sup>th</sup> centile (Goetzinger et al. 2012b, Mathiesen et al. 2014, Ventura et al. 2012, Weisz et al. 2007), <10<sup>th</sup> centile (Goetzinger et al. 2012b, Todros et al. 2004, Özlü and Ozcan 2013), <-1.5 SD (Fukada et al. 1997) and –2 SD (Zalel et al. 2002) for specific gw have been used. SF has also been defined as a BPD/FL ratio >1.5 SD (Lockwood et al. 1987) or as an observed/expected– bone length ratio <0.90 (Bahado-Singh et al. 2002) or <0.91 (Bottalico et al. 2009), where the expected FL is based on the BPD of the fetus.

In previous studies, 0.3–1.8% of screened fetuses had an SF (<5<sup>th</sup> centile) in the second trimester US (de Carvalho et al. 2013, Goetzinger et al. 2012b, Mathiesen et al. 2014), while it was present in 16.2% of fetuses with DS (Mathiesen et al. 2014). The likelihood ratios (LR) for DS in previous studies are variable, from up to 4.31 in previously screened populations (Aagaard-Tillery et al. 2009) to up to 55.0 in high-risk populations (Wax et al. 2000).

Several studies have reported an association of SH with DS (Bromley et al. 2002b, Gray et al. 2009). In DS detection, many authors consider SH to be superior to SF. The definitions for SH in studies are also variable and similar to those used with SF. In the study by Gray et al. (2009), SH <5<sup>th</sup> centile was found to have the best screening performance for DS. In the same study, 9.4% of fetuses with DS had SH (<5<sup>th</sup> centile) compared to 1.5% of euploid fetuses. LRs for
DS in the literature vary from up to 5.56 in previously screened populations (Schluter and Pritchard 2005) to up to 36.76 in high-risk populations (Wax et al. 2000).

\textit{Pyelectasis}

In the late 1980s, the fetal renal pelvic anteroposterior dilatation called renal pyelectasis (PY) was studied concerning the risk of significant renal disease. In the process, a higher than expected incidence of DS was found among fetuses with mild PY (Benacerraf et al. 1990b) and since then, more research concerning this association has been carried out. In most studies, PY is defined as an anteroposterior diameter of 3-5 mm (Aagaard-Tillery et al. 2009, Carbone et al. 2011, Weisz et al. 2007); additionally, gestational age-specific cut-off levels (4 mm at 16-20 weeks, 5 mm at 20-30 weeks, 7 mm at 30-40 weeks) have been used (Shipp and Benacerraf 2002). Isolated PY is seen in 1-3\% of fetuses during the second trimester (Carbone et al. 2011, Coco and Jeanty 2005) and the incidence among fetuses with DS is 17-25\% (Shipp and Benacerraf 2002). The LR for DS has been estimated to be 2.44-17.44 (Bottalico et al. 2009, Carbone et al. 2011), but controversy exists concerning the significance of isolated PY as a soft marker (Carbone et al. 2011, Chudleigh et al. 2001, Coco and Jeanty 2005, Orzechowski and Berghella 2013, Smith-Bindman et al. 2007). PY rarely has other prenatal or postnatal pathologic significance, but the risk increases with the degree of pelvic dilatation (Rao and Platt 2016).

\textit{Echogenic bowel}

After the study by Nyberg et al. (1990), the next US marker associated with DS was echogenic fetal bowel (EB). It is a soft marker defined as an increased echogenicity of the fetal bowel, equal to that of bone, or with increasing echogenicity in grades 0-3 (Nyberg and Souter 2001b). The incidence of EB in the second trimester US is estimated to be 0.2-7\% (Rao and Platt 2016, Nyberg et al. 1990) and LRs for DS of up to 38.6 in previously screened populations have been reported (Weisz et al. 2007). However, the clinical significance of isolated EB is unclear (Bethune 2007a, Mailath-Pokorny et al. 2012).

Other adverse outcomes in pregnancies with EB have also been reported and the risk of IUGR and SB has been shown to be increased (Goetzinger et al. 2011). Additionally, associations with congenital infections, intra-amniotic bleeding, fetal cystic fibrosis, early anemia or
hydrops and primary gastrointestinal abnormalities have been reported (Buiter et al. 2013, Rao and Platt 2016).

**Echogenic intracardial focus**

The echogenic intracardial focus (EIF) is a soft marker originally described as a papillary muscle microcalcification seen in 16% of fetuses with DS (Roberts and Genest 1992). It is the most commonly seen soft marker and is defined as an echogenic structure in the fetal heart. It is usually small, but can be up to 4-6 mm in size and with an echogenicity comparable to bone (Altug and Danisman 2013, Rodriguez et al. 2013). EIF is most commonly seen in the left ventricle (Gupta et al. 2010, Shakoor et al. 2013), but can be bilateral, right-sided or multiple.

EIF is estimated to occur in 3-5 % of low-risk pregnancies (Huang et al. 2010, Merati et al. 1996), but in up to 30% of trisomic pregnancies (Sotiriadis et al. 2003). The association with DS is disputable and some authors consider EIF a normal variant, especially when seen in isolation (Anderson and Jyoti 2003, Bethune 2007b, Bradley et al. 2005, Filly et al. 2004b, Ouzounian et al. 2007, Petrikovsky et al. 2005, Rodriguez et al. 2013). However, a positive association and a five-to seven-fold-risk of DS has also been reported (Sotiriadis et al. 2003, Winter et al. 2000). An increased risk of DS has also been reported in pregnancies with isolated EIF (Coco et al. 2004, Nyberg and Souter 2001b, Winter et al. 2000). Thus, the extent of association with DS is unclear (Bethune 2007b). The exact etiology of EIF is unknown (Altug and Danisman 2013), and it usually disappears in late pregnancy or early infancy. No association with major cardiac abnormalities has been detected (Degani et al. 2001, Merati et al. 1996, Wolman et al. 2000).

**Choroid plexus cyst**

The choroid plexus tissue in the brain is responsible for production of cerebrospinal fluid. During the development of the brain, an outpouching of fluid can be seen as a choroid plexus cyst (CPC) in approximately 1-3.6% of fetuses during the second trimester (Chitty et al. 1998, DiPietro et al. 2006, Morcos et al. 1998, Sahinoglu et al. 2004). CPC has been associated with trisomy 18, since 30-50% fetuses with trisomy 18 have been shown to have CPC (Bromley et al. 1996). This has been reported in many studies (Bronsteen et al. 2004, DeVore 2000a, Sahinoglu et al. 2004) and the LR for trisomy 18 has been estimated to be 7.09-39.7 (Choong
Definitions concerning the minimum size of CPC are variable, but a size of at least 5 mm is commonly used (Åhman et al. 2014, Bethune 2008, Chitty et al. 1998). Some authors consider an isolated CPC to be a benign finding in low-risk women (Filly et al. 2004b) and see no association with aneuploidies (Lopez and Reich 2006). CPCs almost always disappear and lack any association with CNS abnormalities (DiPietro et al. 2006).

*Mild ventriculomegaly*

Mild ventriculomegaly (MVM) is among the most common CNS abnormalities seen in US during pregnancy. It is defined as a lateral ventricular measurement >10 mm or 10-15 mm from the axial plane at the level of thalamic nuclei and the posterior portion of the choroid plexus (Aagaard-Tillery et al. 2009, Agathokleous et al. 2013, Chu et al. 2016). In a non-selected population-based study, the MVM was seen in 0.6/1000 pregnancies, and in earlier studies it was associated with an increased risk of DS (Pilu et al. 1999). On the other hand, a more recent analysis found no association with aneuploidies (Scala et al. 2016). The role of MVM as a soft marker is controversial and some authors consider it a normal variant (Nyberg and Souter 2001b). Some, however, have categorized it as a major marker due to the increased risk of associated CNS abnormalities, congenital infections, neurodevelopmental delay or progression during the pregnancy (Chu et al. 2016, Papp et al. 2007, Scala et al. 2016).

*Single umbilical artery and new markers for DS*

Single umbilical artery (i.e., an umbilical cord with one of the two arteries missing) has been associated with aneuploidies. Some studies have used it as a marker for DS, although more recent studies have not reported an increased risk for DS (Chou et al. 2009).

Along with the technological developments, even the smallest features of DS have become visible during pregnancy. Small ears, a widened iliac bone angle, hypoplasia or absence of the fifth finger middle phalanx, a simian crease and separation of the great toe are potentially detectable during pregnancy (Benacerraf et al. 1988, Benacerraf 2010a, Chou et al. 2009, Shipp et al. 1998). However, these subtle markers are either not specific or sensitive enough,
or they are technically too difficult to be used at regular second trimester screening. An aberrant right subclavian artery (ARSA) appears to be a clinically useful marker and, compared to 1.02% in euploid fetuses, is seen in 23.6% of fetuses with DS (Scala et al. 2015, Willruth et al. 2012, Zalel et al. 2008). Although a promising marker, the evaluation of ARSA might be technically too demanding as part of the routine US screening. The commonly used soft markers are shown in Figure 3 and their prevalence among euploid and DS population in Table 1. Ultrasound pictures of the most common soft markers are represented in the Appendix.

Figure 3. Soft markers used in studies of screening for Down syndrome
Table 1. Most common soft markers and their prevalence in euploid and down syndrome population

<table>
<thead>
<tr>
<th>Soft marker</th>
<th>Prevalence in euploid population</th>
<th>Prevalence in Down syndrome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal hypoplasia &lt;0.75MoM</td>
<td>2.8%</td>
<td>33.6-47%</td>
<td>Bahado-Singh et al. 2002, Sonek et al. 2006</td>
</tr>
<tr>
<td>Nuchal fold &gt;6mm</td>
<td>0.1-0.5%</td>
<td>12-42.3%</td>
<td>Bahado-Singh et al. 2002, Bromley et al. 2002, Odibo et al. 2008, Shipp and Benacerraf 2002</td>
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<tr>
<td>Short fetal femur (O/E &lt;0.91)</td>
<td>5.3%</td>
<td>0.5%</td>
<td>Bromley et al. 2002, Wax et al. 2000</td>
</tr>
<tr>
<td>Short fetal humerus (O/E &lt;0.9)</td>
<td>2.1%</td>
<td>0.4-53.7%</td>
<td>Bromley et al. 2002, Wax et al. 2000</td>
</tr>
<tr>
<td>Echogenic bowel</td>
<td>0.4-1.8%</td>
<td>0.8-15%</td>
<td>Bromley et al. 2002, Mailath-Pohorny et al. 2012, Wax et al. 2000, Bethune 2007a</td>
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</table>

MoM=Multiple of Median
Studies concerning the use of soft markers, their combinations and definitions vary widely. Even practices within countries are variable. In our institution, the present guidelines have been used since June 2006. They are presented in Figure 4.

**Figure 4. Second trimester soft markers in Helsinki University Hospital**

**SOFT ULTRASOUND MARKERS IN PRENATAL SCREENING**
Helsinki University Hospital

Presence of two or more of the following soft markers in the second trimester ultrasound (18-22 gw) is an indication for referral and counseling in the Fetal Medicine Unit.

1. Choroid plexus cyst ≥ 5 mm (isolated or multiple)
2. Echogenic intracardial focus (isolated or multiple, regardless of size)
3. Bilateral pyelectasia, 6.0-9.9 mm
4. Echogenic bowel (echogenicity equal to bone)
5. Short fetal femur, ≤ 3rd centile (according to the table)
6. Short fetal humerus, ≤ 3rd centile (according to the table)

<table>
<thead>
<tr>
<th>Gestational age*</th>
<th>Humerus (mm)</th>
<th>Femur (mm)</th>
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<tbody>
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<tr>
<td>21+6</td>
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</table>

*gestational age is determined by the last menstrual period or by the crown-rump length (cases of gestational age discrepancy of more than four days at the time of the first trimester ultrasound).
Different opinions concerning pregnancies with isolated soft markers have been presented. Some authors suggest discussing them even with patients with low a priori risk (Doubilet et al. 2004), while others would not inform patients about a slight shift of estimated risk, if the risk remains low (Filly et al. 2004b). In our institution, patients are not necessarily informed about isolated soft marker findings.

A wide variation of soft markers and their definitions has been used in previous studies. The study populations in the second trimester screening has also been variable and the method and combinations of soft markers different. Table 2 shows some examples of previous studies, the screening population used, sensitivities and soft marker combinations used in these studies.
<table>
<thead>
<tr>
<th>Phalanx on the index finger</th>
<th>Normal population</th>
<th>Pera et al. 2007</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent nasal bone, normal edema, E&lt; 6'2'</td>
<td>39%</td>
<td>0.02% (1/22)</td>
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<tr>
<td>Phalanx on the ring finger</td>
<td>Normal population</td>
<td>Boll et al. 2009</td>
</tr>
<tr>
<td>NF SH ≤ 6'2&quot;; EF ≥ 6'2; Evidence of the phalanx middle</td>
<td>86.6% (8/11)</td>
<td>High risk and low risk</td>
</tr>
<tr>
<td>Phalanx on the middle finger</td>
<td>Normal population</td>
<td>Kikuta et al. 1999</td>
</tr>
<tr>
<td>NF SH ≤ 6'2&quot;; EF ≥ 6'2; Evidence of the phalanx of the middle finger</td>
<td>92% (32/35)</td>
<td>High risk and low risk</td>
</tr>
<tr>
<td>NF SH ≤ 6'2&quot;; EF ≥ 6'2; Evidence of the phalanx of the index finger</td>
<td>92% (32/35)</td>
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Table 2: Examples of previous studies using set markers in screening for Down syndrome.
Screening for adverse pregnancy outcome

Screening for structural abnormalities

In most countries with up-to-date care for pregnant women, both first and second trimester US screenings are provided. The second trimester US is a systematic examination of fetal anatomy and the location of the placenta, primarily at 18\(^{+0-21^{+6}}\) weeks of pregnancy. The purpose of the exam is identification of severe congenital abnormalities in the fetal CNS, heart, limbs, gastrointestinal and urinary tracts and the improvement of neonatal outcomes.

The time frame represents a balance between appropriate visibility of the fetal structures and the possible residual time for further investigations, if a fetal abnormality is suspected. In Finland, TOP for fetal indications is not possible after 24 weeks of pregnancy. However, screening for structural abnormalities can be performed after 24 weeks of pregnancy, when TOP is no longer an option. The purpose of this screening is to plan the treatment of the newborn or mother if abnormalities are detected.

Screening for other adverse pregnancy outcomes

Second trimester screening for DS may be performed as a triple screen (AFP, \(uE3\), and total hCG), or a quadruple screen by using dimeric inhibin-A simultaneously with the aforementioned markers.

In the absence of chromosomal abnormalities, elevation of maternal serum AFP (>2.5 MoM) is associated with fetal open NTDs and other severe fetal structural abnormalities (Killam et al. 1991). It has also been associated with poor pregnancy outcomes, including SA, SB, placental abruption, gestational hypertension with proteinuria (Shenhav et al. 2002) and PTD, however, only in combination with other markers, not independently (Wenström et al. 1996).

Abnormal placentation has also been reported (Hung et al. 1999, Koster et al. 2004). Higher levels seem to correlate with higher risk of poor outcomes (Cusick et al. 1996, Killam et al. 1991), but low maternal serum AFP (<0.25 MoM) has also been associated with SA (Doran et
al. 1987, Krause et al. 2001), PTD (Krause et al. 2001), SB (Burton 1988, Krause et al. 2001), infant death and increased risk of macrosomia (Baschat et al. 2002, Kiran et al. 2005). The exact cause for the elevation of AFP is not fully understood, but studies suggest an underlying explanation in chorionic villitis and placental vascular lesions (Salafia et al. 1988). These placental lesions or the fetal structural defect allow the leakage of AFP from high fetal concentrations to maternal circulation.

An increased hCG-level in the second trimester of pregnancy is assumed to be associated with hypoxia-induced cytotrophoblast proliferation and an increase in hCG production (Lieppman et al. 1993). Chorangiosis (Stroustrup-Smith et al. 2003) and placental mosaicism (Morssink et al. 1996) has been reported. Other than chromosomal abnormalities, high levels of hCG appear to be associated with fetal growth restriction (FGR) (Heinonen et al. 1996, Lepage et al. 2003), gestational hypertension with proteinuria (Lepage et al. 2003, Shenhav et al. 2003), PTD (Hershkovitz et al. 2003, Lieppman et al. 1993) and SB (Walton et al. 1999). Low levels of hCG in the second trimester do not increase the risk of poor pregnancy outcomes (Spencer et al. 2005).

Low values (≤0.5 MoM) of uE3 have been associated with FGR, SB and pregnancy loss (Dugoff et al. 2005, Kowalczyk et al. 1998, Schleifer et al. 1995) and high levels with triploidic pregnancies, HELLP syndrome (Hemolysis, Elevated Liver Enzymes, Low Platelet) and loss of one twin in the first trimester (Goodwin et al. 2002). High uE3 or low inhibin-A levels are not associated with obstetric complications. The second trimester screening in HUH does not include uE3 or inhibin-A.

**SFLB as a marker of adverse outcome**

In the 1990s, it become apparent that fetuses can present a SF in the second trimester of pregnancy as an early feature of FGR (Bromley et al. 1993, O'Brien and Queenan 1982, Pattarelli et al. 1990). Apart from the association with DS, poor fetal growth was detected to be the most common adverse outcome associated with SF (Papageorghiou et al. 2008, Todros et al. 2004, Ventura et al. 2012, Weisz et al. 2008).
Definitions for SF (<5<sup>th</sup> centile, <10<sup>th</sup> centile, -2 SD) and poor fetal growth in the literature vary considerably and thus the results of studies are not easily comparable. Some studies have used SGA assessed by low birthweight only (birthweight <10<sup>th</sup> centile, <-2 SD) (Mathiesen et al. 2014, Ventura et al. 2012, Weisz et al. 2008), and some assessed by low estimated fetal weight (EFW) (<10<sup>th</sup> centile) together with abnormal umbilical artery Doppler (UmaD) (de Carvalho et al. 2013). In some studies, SGA is assessed by abdominal circumference <10<sup>th</sup> centile (Todros et al. 2004) or <5<sup>th</sup> centile together with an abnormal UmaD (Papageorghiou et al. 2008). Due to different definitions of fetal growth, the results of previous studies may include some overlap of constitutionally small and growth restricted fetuses. However, they have shown a remarkable number (13.9-43%) of fetuses with second trimester SF being born SGA or growth restricted (de Carvalho et al. 2013, Goetzinger et al. 2012b, Mathiesen et al. 2014, Nyberg 2008, Papageorghiou et al. 2008, Todros et al. 2004, Ventura et al. 2012, Vermeer and Bekker 2013, Weisz et al. 2008).

The incidence of SH in the study by de Carvalho et al. (2013) was 6.2%. Compared to SF, it was seen more often in pregnancies with FGR and the association was stronger (de Carvalho et al. 2013, Ventura et al. 2012, Weisz et al. 2008). In another study, growth restricted fetuses at 30-31 weeks of gestation had significantly shorter humeri than fetuses with normal growth. FLs did not differ between the groups (Larciprete et al. 2005). In the small study by de Carvalho et al. (2013), 1.4% of fetuses had both SF and SH. FGR (<10<sup>th</sup> centile + UmaD pulsatility index (PI) >95<sup>th</sup> centile) was detected in 33.3% of fetuses with both SF and SH (de Carvalho et al. 2013).

An association of SF with PE has been suggested in some studies (Papageorghiou et al. 2008, Todros et al. 2004), but opposing results have also been reported (Goetzinger et al. 2012b, Ventura et al. 2012, Vermeer and Bekker 2013, Weisz et al. 2008). Results concerning the association of SF with PTD (Goetzinger et al. 2012b, Todros et al. 2004, Weisz et al. 2008, Özlü and Ozcan 2013) and with the risk of an Apgar score <7 at 5 min (Ventura et al. 2012, Weisz et al. 2008, Özlü and Ozcan 2013) are conflicting, but the risk of SB (Goetzinger et al. 2012b, Mathiesen et al. 2014) and MC have been shown to be increased (Mathiesen et al. 2014).

Several etiological explanations for SFLB have been presented and the role of underlying placental insufficiency highlighted (Todros et al. 2004, Zalel et al. 2002). Due to abnormal
placentation, the level of fibroblast growth factor 2 increases and decreases the growth plate chondrocyte proliferation, cellular hypertrophy, and, at high concentrations, cartilage matrix formation of the fetal long bones (Mancilla et al. 1998, Zalel et al. 2002). Through the IGF system, PAPP-A is also linked to bone development, skeletal growth (Conover et al. 1999, Lawrence et al. 1999, Leung et al. 2006, Peterson and Simhan 2008, Prefumo et al. 2006, Qin et al. 2006) and SFs (Weisz et al. 2008). Maternal serum PAPP-A levels at 10-14 gw are significantly associated with the length of fetal humeri, femurs and tibias (Peterson and Simhan 2008).

Another possible etiology was described back in 1970s. SFLB might be explained by an early vascular adaptation to placental dysfunction and chronic hypoxia. At the expense of extremities, the oxygenated fetal blood is shunted toward more vital organs such as the brain, heart and lungs (Peeters et al. 1979, Sheldon et al. 1979, Weisz et al. 2008). Abnormal placental sonographic findings, including infarcts, edema, and chorangiosis, increase the number of syncytial knots, and calcification has been described in pregnancies with SFs (Zalel et al. 2002).

Maternal height, weight, parity and ethnic background have to be taken into account when assessing the fetal long bone measurements. Customized growth charts are helpful in ethnically-heterogenic populations (Drooger et al. 2005, Gardosi et al. 1992) in order to minimize FPRs.

Skeletal dysplasias are a wide spectrum of diseases characterized by SFLB. They are almost always accompanied by additional US abnormalities such as a narrow thorax, protuberant abdomen and anomalies of the skull (Parilla et al. 2003). The bone length is usually very short and bones are abnormal in shape (Alanay et al. 2007, Papageorghiou et al. 2008, Todros et al. 2004). Fetuses with skeletal dysplasia very rarely represent isolated long bone shortening (Schramm et al. 2009, Trujillo-Tiebas et al. 2009).
Positive screening result and karyotyping

Karyotyping and genetic counseling are offered in cases of an NT $\geq 3$ mm or a combined FTS risk of $\geq 1/250$ for DS or $\geq 1/100$ for trisomy 18. An option for an NIPT or, depending on the gestation of the pregnancy, either chorionic villus sample (CVS) or amniocentesis (AC) is offered.

In HUH, NIPT has been used since the beginning of 2015. It involves an analysis of cell-free fetal deoxyribonucleic acid (cfDNA) from maternal blood. Most of the common trisomies can be analyzed by NIPT and it is extremely specific (99%) and sensitive (99.9%) for trisomy 21. The specificities for trisomies 13 and 18 are also good, 91% and 96% respectively, and aberrations of the sex chromosomes can also be screened for (Alberti et al. 2015, Gil et al. 2015, Liang et al. 2013, Porreco et al. 2014, Verweij et al. 2013, Zhang et al. 2015). Some cases of FPRs have been reported (Dugo et al. 2014), and a positive test has to be confirmed by an invasive test, either CVS or AC. However, compared to the 3% PPV of combined FTS, the PPV of NIPT has been reported to be significantly better (81%).

Some women initially opt for an invasive test and two techniques are used. AC was introduced in 1956 by Fuchs and Riis (Crandall and Sparkes 1973, Fuchs and Riis 1956) and the first US-guided CVS in 1984 (Smidt-Jensen and Hahnemann 1984, Smidt-Jensen et al. 1986). CVS can be used between 10 and 13 gw to obtain fetal cells from the placenta for chromosomal analysis. Placental cytotrophoblasts are rapidly duplicating and rich in deoxyribonucleic acid (DNA). Due to the rapid duplication, the sample does not necessarily need to be cultured. The analysis of most common trisomies takes up to one week. This technique is not possible in cases of posterior placenta in a retroverted uterus.

AC can be used from 14 gw onward. Amniotic fluid contains cells from fetal skin, lungs, bladder and the amnion. Cells need to be cultured and the final analysis takes approximately 14 days. In 1-2% of samples, the CVS result shows placental mosaicism and the result has to be confirmed by AC, which usually contains only fetal cells (Hahnemann and Vejerslev 1997).
A polymerase chain reaction (PCR)-based test is usually applied to assess the karyotype. It is rapid and able to detect trisomies 21, 18 and 13, as well as sex chromosome abnormalities. Full cytogenetic karyotyping is offered in cases with a NT of at least 4 mm.

Generally, the procedure-related risk of fetal loss has been considered to be 0.5-1.0% (Alfirevic et al. 2003, Tabor et al. 1986), but lower risk figures for both CVS (0.06-0.1%) and AC (0.2%) (Akolekar et al. 2011, Akolekar et al. 2015, Eddleman et al. 2006), as well as no increased risk (Wulff et al. 2016), have been reported recently. The risk has been shown to be related to physicians technical experience (Tabor et al. 2009, Verjaal et al. 1981).

The chromosomal microarray technique is able to detect small, submicroscopic changes in the DNA. Compared to conventional karyotyping, the microarray technique has been better in detecting duplications or deleted sections of DNA in fetuses with no structural abnormalities detected in the US (Wapner et al. 2012) and in mentally retarded people with normal karyotypes (Siggberg et al. 2010). Sometimes however, an abnormal microarray result with variants of unknown significance is challenging in counseling parents (Mikhaelian et al. 2013), but sufficient clinical experience reduces this problem.

The management of pregnancies with abnormal screening results or other high-risk attributes is presented in Figure 5.
Figure 5. The management in cases of abnormal screening result or other risk

*women not opting for invasive testing / termination of pregnancy under any circumstances,

** trisomy-PCR and sex-chromosomes, *** trisomy-PCR and sex-chromosomes, if normal aCGH

FTS=first trimester screening, NT=nuchal translucency, IUGR=intrauterine growth restriction, US=ultrasound, NIPT=non-invasive prenatal test, CVS=chorionic villus sample, gw=gestational weeks, AC=amniocentesis, PCR=quantitative fluorescence polymerase chain reaction, aCGH=array comparative genomic hybridization
Combinations of different prenatal screening strategies

In an attempt to increase the DR for DS, several combinations of serum screening methods have been introduced. In the integrated screening strategy, the risk is calculated only after the second trimester screening, and the result is based on both the first and the second trimester screening results. In independent sequential screening, the first and second trimester risks are calculated independently, and in the step-wise sequential protocol, the second trimester screening is offered to all patients with a negative result in the combined FTS. In the contingent screening method, patients are divided into screen-positive, screen-negative or an intermediate group based on the combined FTS result. Second trimester screening is offered only to patients in the intermediate group.

A study by Cuckle et al. (2008) reported a 91% DR with a 4.5% FPR for the contingent method, a 92% DR with a 5.1% FPR for the step-wise method and an 88% DR with a 4.9% FPR for the integrated method. A final risk > 1/270 was considered to be screen-positive and the optimal contingent screening result was achieved when defining intermediate risk, 1/30-1/1300.

Different methods have also been used when evaluations of the significance of second trimester US markers and outcomes of previous research are variable. Until 1998, soft marker studies mostly included high-risk populations (i.e., mothers with advanced age, at high risk in second trimester serum screening or a previous affected pregnancy).

A scoring index system was introduced to evaluate the risk for DS in high-risk population after second trimester US (Benacerraf et al. 1992, Bromley et al. 1999, Bromley and Benacerraf 2003). A score was assigned for each fetus (score 2=major anomaly, NF≥6mm, score 1=SF, SH, PY, EIF, CPC) and a score ≥1 was an indication for karyotyping. A DR of 62.5% for DS was reported. If women ≥40 years of age were offered karyotyping, the DR increased to 75%.

In 1998, an age-adjusted US risk assessment was developed by Nyberg et al (1998). An LR for DS was given to all soft markers and the risk was assessed based on maternal age, serum markers and second trimester US (Nyberg et al. 1998). After the second trimester US, the background risk is modified, taking into account all previous screening test results. Some authors have even used negative LRs to reduce the individual risk after normal second
trimester US (Vintzileos et al. 2002), but the reduction of risk is not approved by all authors (Lau and Evans 2008).

Since up to 90% of DS cases are already detected in the first trimester, the second trimester screening has significantly changed after the introduction of soft markers. This raises questions about the usefulness of soft markers in the second trimester US. The study by Sood et al. (2009) evaluated the use of soft markers after FTS and found it useful in patients with high risk in the combined FTS, but of limited value in low-risk patients. On the other hand, the study by Krantz et al. (2007) reported an additional 6.1% of DS cases with 1.2% FPR after normal combined FTS.

**Placental dysfunction and the significance of normal fetal growth**

Poor fetal growth is associated with increased morbidity and mortality. Different terms are used to describe low EFW, and some confusing overlap exists.

The term small for gestational age (SGA) is used when the EFW is less that a certain threshold, usually 5th or 10th centile, at a certain gestational age. A significant proportion of these fetuses are constitutionally small due to genetic factors including ethnicity, maternal weight and height. These fetuses are not at an increased risk for obstetric complications.

When the fetus is not fulfilling its growth potential, the condition is called intrauterine growth restriction (IUGR). The most common reasons are chromosomal and structural abnormalities, placental dysfunction and infections. According to the time of onset of IUGR, both early and late IUGR are described with different histological, biochemical and clinical features (Crispi et al. 2006). Early onset IUGR is often diagnosed with an abnormal UmaD and is frequently associated with PE. These abnormalities are less frequently seen in late onset IUGR.
The distinction of these situations is valuable when evaluating the need for further investigations and assessing the risk of obstetric or neonatal complications. IUGR, apart from SGA, increases perinatal morbidity and mortality due to an underlying exposure to intrauterine hypoxia and subsequent PTD (Bashat et al. 2007, Bernstein et al. 2000, Damodaram et al. 2011, Gardosi 2014, Kady and Gardosi 2004, Platz and Newman 2008). Perinatal morbidity is linked with the length of impaired growth and increases according to the length of poor growth (Illa et al. 2009). The risk of long-term neurological and developmental deficits is also increased (de Bie et al. 2010, Doctor et al. 2001), as well as the risk of hypertension, hyperlipidemia, coronary heart disease and diabetes mellitus in adulthood (Barker 2006). The risk of Caesarean section (CS) and neonatal intensive care unit admission are also increased in these pregnancies (Vermeer and Bekker 2013). When excluding congenital anomalies, potentially more than 50% of SBs have preceding growth restriction (Gardosi et al. 2005, Vergani et al. 2008). The risk of dying in utero is 5-10–fold increased when the fetus does not fulfill its growth potential (Clausson et al. 2001).


Since no treatment has been shown to be beneficial for IUGR (Gulmezoglu & Hofmeyr 2000, Gulmezoglu & Hofmeyr 2001, Laurin & Persson 1987, Say et al. 2003), optimal timing of delivery remains the only treatment. The identification of pregnancies with IUGR and a structured surveillance, however, reduces the risk of adverse pregnancy outcomes (Linqvist and Molin 2005).
Ethics of screening

In general, screening is acceptable if a treatment for the screened condition exists. This is not the case in prenatal screening for chromosomal abnormalities, since neither prevention nor treatment for aneuploidies exists. However, improvements in healthcare and social acceptance have significantly improved the life expectancy and quality of life for individuals with DS over the last few decades (Covelli et al. 2016, van Gameren-Oosterom et al. 2011).

Prenatal screening is justified based on the pregnant woman’s autonomy concerning the pregnancy. In cases with diagnosed aneuploidy or severe structural abnormality, women and parents have an option to either continue the pregnancy or to choose TOP. In 2012, 3.4% of all TOPs in Finland were made due to fetal abnormality (http://www.julkari.fi/bitstream/handle/10024/110210/Tr18_13.pdf?sequence=5). Even though, the pregnant women’s autonomy is a fundamental right, prenatal screening has been criticized as being a judgement against the disabled and a threat to human rights. This criticism sometimes emerges as an accusation, and screening has been seen as part of eugenic societal behavior (Gillott 2001).

When an increased risk for chromosomal abnormality is detected, parents can opt for a diagnostic test (CVS or AC). However, all screening tests carry a risk of FPR and the procedure-related risk is approximately 0.5%. The increasing use of cffDNA screening for most common fetal aneuploidies, as well as whole genome sequencing, removes the risk of karyotyping. However, it brings new ethical challenges to pregnant women, as well as the healthcare providers.

The anxiety concerning chromosomal abnormalities is usually relieved with a normal test result (Lou et al. 2015). However, the anxiety caused by soft marker detected in the second trimester screening has been shown to continue until delivery and sometimes even longer, altering the interaction between mother and infant. False negative results are also possible in screening and all conditions are not diagnosed during pregnancy, but will be detected only after birth. This causes depression and anxiety, as well as bitterness and more negative attitudes toward the child (Hall et al. 2000).
Parental counseling

When attending prenatal screening, most women seek reassurance that everything is normal (Åhman et al. 2010, Kohut et al. 2002). It is natural to hope for a healthy baby and not to consider other options. This may, however, affect the interest in the pre-screening information that is given.

More commonly than believed, women lack information about the purpose of US examinations (Ekelin et al. 2004, Eurenius et al. 1997, Lalor and Devane et al. 2007), and information concerning prenatal screening and second trimester US is often inadequately understood (Cash et al. 2010, Kohut et al. 2002). Many women see the prenatal US more like a social than a medical event (Åhman et al. 2010). In the second trimester US, most women wish to be informed about soft markers, yet a minority of them understand the information they might provide (Cash et al. 2010, Watson et al. 2002, Åhman et al. 2010). The prospect of a fetal abnormality is a very emotional and sensitive topic to discuss during pregnancy and too much information before the screening is also considered to cause unnecessary anxiety (Lalor and Begley 2006, Åhman et al. 2010). Some women have concerns about fetal abnormality, but only dare to report or discuss them after delivery (Watson et al. 2002).

The respect for patient autonomy is the main principle of prenatal screening. True autonomy is only achieved when sufficient pre-screening information is provided with information about the purpose, advantages, risks, limitations and implications of screening. The information given must be free of persuasion, directives and the providers own opinions. After counseling, the evaluation and comprehension of the information given, an informed choice can be made concerning the participation in prenatal screening (Kohut et al. 2002).
AIMS OF THE STUDY

The main goal of this study was to provide data for parental counseling by evaluating the significance of second trimester soft US markers, particularly SFLB, after normal FTS in fetuses without structural abnormalities. Another goal was to evaluate the significance of low maternal first trimester PAPP-A levels as an independent marker of adverse pregnancy outcomes.

The specific aims of the four studies were:

1. To evaluate the prevalence of aneuploidies in pregnancies with a normal FTS and two or more second trimester soft markers detected in fetuses without structural abnormalities (Study I).
2. To evaluate the outcome of pregnancies with a normal FTS and isolated SFLB detected in the second trimester US in fetuses without structural abnormalities (Study II).
3. To evaluate and compare the outcome of pregnancies with extremely low and normal levels of first trimester PAPP-A (Study III).
4. To evaluate and compare the outcome of pregnancies in three subgroups with extremely low first trimester PAPP-A levels (Study IV).
MATERIAL AND METHODS

Principles of the FTS

The FTS was performed according to the guidelines of the Finnish Ministry of Social Affairs and Health (Health Care Act 339/2011). US was performed according to the Fetal Medicine Foundation protocol and by a sonographer, certified midwife or a specialist in perinatology at the HUH or a HUH District Hospital. Gestational age was determined by the last menstrual period or by the CRL, which was used in case of a gestational age discrepancy of more than four days at the time of the first trimester US. Until the beginning of 2008, the primary chromosomal screening method in the first trimester was NT measurement; later, a combined FTS was offered to all pregnant women. A NT measurement <3 mm or a combined FTS risk <1/250 were considered low risk for DS.

Principles of the second trimester US

The second trimester US was performed by a sonographer, a certified midwife or a specialist in perinatology. A detailed evaluation of fetal anatomy was performed and six soft markers of aneuploidy were used: CPC, EIF, PY, EB, SF and SH. The definition for CPC was a well-defined cyst at least 5 mm in size; EIF was defined as a bright focus in the fetal heart. PY was defined as a bilateral minor dilatation of the renal pelvis (≥6.0-9.9 mm), and the definition for EB was an increased echogenicity equal to bone. SF and SH were defined as gestational age-specific bone length ≤3rd centile with a normal bone morphology.
The significance of soft markers after normal FTS (Study I)

Over a five-year study period (1 JAN 2006-31 DEC 2010), 295 pregnancies with two or more soft markers detected in an otherwise normal second trimester US were referred for detailed evaluation to the Fetal Medicine Unit (FMU) of HUH. All pregnancies had a normal result at the FTS. In the FMU, a detailed US was performed by a specialist in perinatology. All women received counseling and were informed about an aneuploidy risk of 2-3%. Karyotyping with AC was offered and women were informed about a 0.5% risk of AC-related complications. Informed consent was obtained from all women.

Multiple pregnancies, those with no available birth data, and pregnancies with no FTS, abnormal results or previous karyotyping were excluded from the study; the final study group consisted of 228 pregnancies (Figure 6). The rate of aneuploidies was retrospectively analyzed from the hospital’s computer database.

Outcome of euploid pregnancies with SFLB in the second trimester US (Study II)

During the study period (1 JAN 2006-31 AUG 2013), 40 pregnancies with an isolated SFLB detected in an otherwise normal second trimester US were referred for detailed evaluation to the FMU of HUH.

All pregnancies had a normal result at the FTS. In the FMU, a detailed US was performed by a specialist in perinatology to confirm the diagnosis of SFLB. All women received counseling and were informed about an aneuploidy risk of 2-3%. Karyotyping with AC was offered and women were informed about a 0.5% risk of AC-related complications. Informed consent was obtained from all women. Only singleton pregnancies without structural abnormalities, aneuploidy or postnatal features of aneuploidy were included in the study. For each pregnancy, two pregnancies with normal fetal bone lengths were selected as controls. Mothers were age-matched and screened on the same day.
Figure 7. A flow chart indicating the study group inclusion criteria
Pregnancy and perinatal outcomes were retrospectively analyzed by collecting data from hospital’s computer database. The rate of adverse pregnancy outcomes (PTD, SB, SGA, PIH, PE) and absent or reversed end diastolic arteria umbilical flow was recorded, as well as the mode of delivery and the short term neonatal outcome. Placental histology data was collected if available.

PTD was determined as a delivery of a liveborn before 37 gw and SB as a loss of fetal viability after 22 gw and before birth. SGA was determined as a birthweight < -2 SD, and PIH as normal early pregnancy blood pressure (BP) and a BP above 145/95 mmHg at least twice after 20 gw. An increased BP with proteinuria of at least 300 mg/day was determined as PE.

**Outcomes of pregnancies with extremely low PAPP-A during the first trimester (Studies III and IV)**

A retrospective analysis of pregnancies with low PAPP-A in the combined FTS during the study period (1 JAN 2009-31 DEC 2012) was performed.

A cut-off level of 0.3 MoM was set for low PAPP-A. PAPP-A measurements were quantified with time-resolved immuno-fluorometric assays performed using an automatic immuno-analyzer (AutoDELFIA; PerkinElmer, Turku, Finland); the detection limit for PAPP-A was 5 mU/L. The intra-assay coefficient of variation was < 3% and the PAPP-A assay was calibrated against WHO IRP 78/610 standard preparation for pregnancy-specific beta 1-glycoprotein. PAPP-A levels were expressed in age-specific MoMs and were corrected for maternal weight. The Lifecycle Database (PerkinElmer) was used for DS risk assessment.

Genetic counseling and karyotyping were offered to all patients with an increased risk at the combined FTS. Depending on the duration of pregnancy, either CVS or AC was offered for patients opting for a diagnostic test. PCR-based tests for trisomy 21, trisomy 18 and trisomy 13 were used, except in cases with NT ≥ 4 mm, when the fetal karyotype was determine.

All singleton pregnancies with available delivery and pregnancy outcome data were eligible for the study. Adverse pregnancy outcomes, including aneuploidies, structural anomalies, PIH,
PE, SA, PTD, SB and SGA were recorded. PIH was determined as a normal BP in early pregnancy followed by a BP of at least 145/90 mmHg at least twice after 20 gw. PE was determined as proteinuria >300mg/day together with hypertension. A loss of pregnancy or fetal viability before 22 gw was determined as SA, and PTD as any delivery before 37 gw. Fetal death before delivery and after 22 gw was determined as SB, and SGA as birthweight < -2 SD.

In study III, women with PAPP-A levels between 0.9-1.1 MoM were selected as controls. An optimal level of PAPP-A was assumed to minimize the risks attributed to PAPP-A. The control cohort was age- matched and the combined FTS was performed during the same year. In study IV, associations of pregnancy outcome with the level of PAPP-A was evaluated in three subgroups: PAPP-A levels < 0.1 MoM, 0.1-0.199 MoM and 0.2-0.299 MoM. The rates of adverse pregnancy outcomes were collected from the hospital database.

**Statistical analysis**

Statistical analysis was performed using the SPSS 22.0 (Statistical Package for Social Science for Windows, Armonk, NY, USA. IBM Corp.) and a $p$-value <0.05 [95% confidence interval (CI)] was considered statistically significant. Categorial variables were compared by the chi-square test, or Fisher’s exact test when appropriate. Continuous variables were compared by the Mann–Whitney U-test or the Kruskal-Wallis test and a Dunn’s post-hoc test was used in the case of three groups being compared. Odds ratios (OR) with 95% CIs were calculated for different outcomes.

**Ethics**

The study protocols were approved by the Institutional Review Board ($§$ 27/18.8.2011). Approval from the Ethics Committee was not needed.
RESULTS

Chromosomal abnormalities in pregnancies with normal FTS and soft markers detected in the second trimester US (Study I)

The study group consisted of 228 pregnancies (Figure 6) with a normal FTS assessed by a NT measurement (48.5%) or a combined FTS. The mean age of women in the study group was 25.8 (range: 17.8-44.8 years) and the second trimester US was performed, on average, at 19+4 gw (range: 17+5-22+4 gw).

The most common soft marker detected was an EIF, which was detected in 65% of pregnancies. The second most common was CPC, followed by SF (Table 3).

<table>
<thead>
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<th>Soft marker</th>
<th>n (%)</th>
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<tr>
<td>Echogenic intracardial focus</td>
<td>149 (65.4)</td>
</tr>
<tr>
<td>Choroid plexus cyst</td>
<td>108 (47.4)</td>
</tr>
<tr>
<td>Short fetal femur</td>
<td>82 (36.0)</td>
</tr>
<tr>
<td>Short fetal humerus</td>
<td>72 (31.6)</td>
</tr>
<tr>
<td>Pyelectasis</td>
<td>31 (13.6)</td>
</tr>
<tr>
<td>Echogenic bowel</td>
<td>22 (9.6)</td>
</tr>
</tbody>
</table>

After the detailed US and counseling by a perinatology specialist in the FMU, 69.7% of women opted for karyotyping by AC and four cases of aneuploidy were detected. Cases of triploidy and trisomy 18, one each, ended in TOP. In addition, one case of an inversion of chromosome 10 and a case of 4% mosaicism of trisomy 21 were detected. These pregnancies ended in delivery of apparently healthy newborns.

After a normal FTS, the incidence of chromosomal abnormality in our study was 1.8% (4/228). Postnatal features of newborns with no prenatal karyotyping did not reveal any signs of aneuploidy. If cases with inversion of chromosome 10 and 4% mosaicism of trisomy 21 are
excluded, the incidence of the significant chromosomal abnormalities in our study population was 0.9% (2/228) (Figure 8).

Figure 8. Chromosomal abnormalities in pregnancies with soft markers after normal FTS
Pregnancy outcome of euploid fetuses with SFLB in the second trimester US (Study II)

Thirty pregnancies fulfilled the inclusion criteria and 60 age-matched control pregnancies were selected. All patients with SFLB were offered counseling and a detailed US at the FMU and the uptake was 93% (28/30).

There was no difference in mean maternal age. The rate of mothers ≥ 37 years of age, primiparity and smoking in early pregnancy was similar, as well as the mean body mass index. The study and control group characteristics are presented in Table 4.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study group (n=30)</th>
<th>Control group (n=60)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, years</td>
<td>29.7±5.0</td>
<td>29.8±4.1</td>
<td>0.997</td>
</tr>
<tr>
<td>Mothers ≥37 years of age</td>
<td>2 (6.7)</td>
<td>2 (3.3)</td>
<td>0.469</td>
</tr>
<tr>
<td>Primiparity</td>
<td>17 (56.7)</td>
<td>20 (33.3)</td>
<td>0.058</td>
</tr>
<tr>
<td>Smoking</td>
<td>4 (13.3)</td>
<td>7 (11.7)</td>
<td>0.744</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.4±3.3</td>
<td>24.0±4.9</td>
<td>0.785</td>
</tr>
</tbody>
</table>

Values are represented as mean±SD or number (%)
BMI=body mass index

The gestational age, the fetal NT in the FTS and the rate of either combined FTS or the second trimester serum screening was similar in both groups. The gestational age at the second trimester US was also not different between the groups. In the study group pregnancies, fetal femur and humerus lengths were significantly shorter than in the control group pregnancies. Karyotyping was performed in 75% (21/28) of study group pregnancies attending the FMU, constituting 70% of all pregnancies in the study group. Altogether, three patients in the control group underwent karyotyping, one by CVS and two by AC. The indication for CVS was an increased NT, but the indications for AC were not available in the hospital computer database. In cases with no prenatal karyotyping, neither prenatal US nor postnatal examination revealed any features of aneuploidy or structural anomaly. The characteristics of the study and reference population in the first and second trimester screening are presented in Table 5.
Table 5. Characteristics of the study and control group in the first and second trimester screening

<table>
<thead>
<tr>
<th></th>
<th>Study group (n=30)</th>
<th>Control group (n=60)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational weeks at the first trimester US</td>
<td>12.2±0.7</td>
<td>12.0±0.7</td>
<td>0.212</td>
</tr>
<tr>
<td>NT in the first trimester US (mm)</td>
<td>0.94±0.3</td>
<td>1.12±0.5</td>
<td>0.046</td>
</tr>
<tr>
<td>Combined FTS or second trimester serum screening</td>
<td>19 (63)</td>
<td>39 (65)</td>
<td>1.00</td>
</tr>
<tr>
<td>Gestational weeks at the second trimester US</td>
<td>19.9±0.8</td>
<td>19.9±0.7</td>
<td>0.997</td>
</tr>
<tr>
<td>FL in the second trimester US (mm)</td>
<td>26.18±2.2</td>
<td>32.1±2.51</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HL in the second trimester US (mm)</td>
<td>25.8±1.9</td>
<td>30.7±1.9*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Karyotyping</td>
<td>21(70)</td>
<td>3 (5.0)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are represented as mean±SD or number (%)

FTS= first trimester screening, NT=nuchal translucency, FL=femur length, US=ultrasound, HL=humerus length

*missing in 7 cases

One case with loss of fetal viability was recorded in the study group at 23+3 gw. All other cases in the study and reference group proceeded to liveborn delivery. In the study group, fetuses were delivered significantly earlier and the birthweight among all fetuses, and among liveborn fetuses, was significantly smaller (Table 6).
The incidence of PE, umbilical artery flow abnormalities (OR 45.2, 95% CI 5.5-370.0) and prematurity (OR 20.8, 95% CI 6.7-64.4) were significantly higher in the group with SFLB. In one out of 22 (5%) and 4/7 (57%) cases in the study and reference groups, preterm premature rupture of membranes preceded the PTD. These deliveries occurred at 36+1 gw and at 32+4-36+4 gw in the study and reference groups, respectively, and there were no cases of SGA among the newborns. All other cases of PTD were iatrogenic due to fetal or maternal indications.

Significantly more cases of SGA were observed in the study group. Seventeen cases were observed among 26 liveborn (65.4%), and 16/17 (94.1%) of these were delivered preterm. The incidence of emergency CS was also significantly higher among pregnancies with SFLB (OR 11.8, 95% CI 3.9- 35.7) and all cases were preterm deliveries due to maternal or fetal indications. In the control group, 2/6 (33%) cases were preterm.

Three and four cases of SB and perinatal death, respectively, were observed among pregnancies with SFBL compared to no cases in the reference group. In three out of four cases of perinatal death, newborns were severely SGA. In the group with SFLB, the OR for any adverse outcome was 24.9 (95% CI 7.8-79.1), the incidence being as high as 76.6%. Pregnancy outcomes in the study and control groups are presented in Figure 9.

In the study group, placental histology was available in the case with loss of fetal viability, in 2/3 (66%) cases of SB, and in 12/19 (63%) cases with liveborn PTD. In 12/15 (80%) cases, the examination of placental histology revealed remarkable pathological features, mostly ischemia and immaturity. Placental histology results for the available cases are displayed in Table 7.
Figure 9. The outcome of pregnancies in the study and control groups.
Table 7. The placental histology of pregnancies with short femur and short humerus

<table>
<thead>
<tr>
<th>Gestational weeks at birth</th>
<th>Placental histology (principal features)</th>
<th>UmaD</th>
<th>Mode of delivery</th>
<th>Outcome of pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23&lt;sup&gt;1&lt;/sup&gt;</td>
<td>immaturity</td>
<td>-</td>
<td>VD</td>
</tr>
<tr>
<td>2</td>
<td>27&lt;sup&gt;15&lt;/sup&gt;</td>
<td>immaturity, ischemia, placenta circumvallata</td>
<td>AEDF</td>
<td>CS</td>
</tr>
<tr>
<td>3</td>
<td>23&lt;sup&gt;15&lt;/sup&gt;</td>
<td>normal</td>
<td>-</td>
<td>VD</td>
</tr>
<tr>
<td>4</td>
<td>35&lt;sup&gt;15&lt;/sup&gt;</td>
<td>normal</td>
<td>normal</td>
<td>CS</td>
</tr>
<tr>
<td>5</td>
<td>25&lt;sup&gt;15&lt;/sup&gt;</td>
<td>immaturity, chorangiosis, hydrops</td>
<td>AEDF</td>
<td>CS</td>
</tr>
<tr>
<td>6</td>
<td>31&lt;sup&gt;13&lt;/sup&gt;</td>
<td>mild ischemia, strong umbilical cord coiling</td>
<td>normal</td>
<td>CS</td>
</tr>
<tr>
<td>7</td>
<td>31&lt;sup&gt;16&lt;/sup&gt;</td>
<td>ischemia</td>
<td>AEDF</td>
<td>CS</td>
</tr>
<tr>
<td>8</td>
<td>32&lt;sup&gt;15&lt;/sup&gt;</td>
<td>immaturity</td>
<td>REDF</td>
<td>CS</td>
</tr>
<tr>
<td>9</td>
<td>35&lt;sup&gt;12&lt;/sup&gt;</td>
<td>ischemia, hypoplasia, thrombosis</td>
<td>normal</td>
<td>VD</td>
</tr>
<tr>
<td>10</td>
<td>28&lt;sup&gt;13&lt;/sup&gt;</td>
<td>ischemia, placenta circumvallata</td>
<td>AEDF</td>
<td>CS</td>
</tr>
<tr>
<td>11</td>
<td>27&lt;sup&gt;15&lt;/sup&gt;</td>
<td>ischemia</td>
<td>AEDF</td>
<td>CS</td>
</tr>
<tr>
<td>12</td>
<td>25&lt;sup&gt;13&lt;/sup&gt;</td>
<td>ischemia, hypoplasia, infarctus</td>
<td>AEDF</td>
<td>CS</td>
</tr>
<tr>
<td>13</td>
<td>34&lt;sup&gt;15&lt;/sup&gt;</td>
<td>normal</td>
<td>-</td>
<td>VD</td>
</tr>
<tr>
<td>14</td>
<td>26&lt;sup&gt;15&lt;/sup&gt;</td>
<td>ischemia, infarctus</td>
<td>AEDF</td>
<td>CS</td>
</tr>
<tr>
<td>15</td>
<td>28&lt;sup&gt;14&lt;/sup&gt;</td>
<td>multiple infarctus</td>
<td>AEDF</td>
<td>CS</td>
</tr>
</tbody>
</table>

UmaD=umbilical artery Doppler, VD=vaginal delivery, SGA=small for gestational age, SB=stillbirth, AEDF/REDF=absent/reversed end diastolic flow, CS=Caesarean section, PND=perinatal death
Outcomes of pregnancies with low and normal first trimester maternal PAPP-A (studies III and IV)

During the four-year study period, combined FTS results were available for 68,861 pregnancies in the HUH District. PAPP-A < 0.3 MoM was detected in 1.7% of pregnancies (n=1166) and the outcome information was available for 961(82.4%) of those pregnancies.

No difference was observed between the two groups regarding mean maternal age or incidence of primiparous patients, but the incidence of smokers and obesity was higher in the low PAPP-A group. The incidence of maternal type 1 diabetes and pre-existing hypertension requiring medication was similar in both groups (Table 8).

Table 8. Characteristics of the study and control groups

<table>
<thead>
<tr>
<th></th>
<th>PAPP-A &lt;0.3 MoM</th>
<th>PAPP-A 0.9-1.1 MoM</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=961)</td>
<td>(n=961)</td>
<td></td>
</tr>
<tr>
<td>Maternal age, years</td>
<td>30.7±5.4</td>
<td>30.8±5.4</td>
<td>0.596</td>
</tr>
<tr>
<td>Maternal age ≥37 years</td>
<td>115 (12.0)</td>
<td>117 (12.2)</td>
<td>0.889</td>
</tr>
<tr>
<td>Primiparity</td>
<td>357 (37.1)</td>
<td>350 (36.4)</td>
<td>0.741</td>
</tr>
<tr>
<td>Smoking *</td>
<td>158 (16.4)</td>
<td>112 (11.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus type 1</td>
<td>9 (0.9)</td>
<td>6 (0.6)</td>
<td>0.604</td>
</tr>
<tr>
<td>Pre-existing hypertension requiring medication</td>
<td>2 (0.2)</td>
<td>3 (0.3)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>BMI (kg/m²) **</td>
<td>24.7±5.2</td>
<td>24.0±4.6</td>
<td>0.333</td>
</tr>
<tr>
<td>BMI ≥30 (kg/m²)</td>
<td>117 (12.1)</td>
<td>91 (9.5)</td>
<td>0.010</td>
</tr>
</tbody>
</table>

values are expressed as mean±SD or number (%)
PAPP-A=pregnancy associated plasma protein A, MoM=multiple of median, BMI=body mass index
* missing: PAPP-A <0.3 MoM: n=85 (8.8%); PAPP-A 0.9-1.1 MoM:n=2 (0.2%)
** missing: PAPP-A <0.3 MoM: n=92 (9.6%); PAPP-A 0.9-1.1 MoM: n=21 (2.2%)
The mean±SD PAPP-A levels in low PAPP-A and control cohorts were 0.23±0.06 and 1.0±0.65, and the duration of pregnancy was similar at the time of first trimester US screening. There was no difference in mean NT between the groups, but more cases of increased NT (> 3 mm) were observed among patients with low PAPP-A. The mean level of hCG-β was significantly lower and the incidence of an increased risk (>1/250) for DS significantly higher in the group with low PAPP-A (Table 9).

Table 9. Characteristics of the study and control groups in the combined FTS

<table>
<thead>
<tr>
<th></th>
<th>PAPP-A &lt;0.3 MoM (n=961)</th>
<th>PAPP-A 0.9-1.1 MoM (n=961)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAPP-A (MoM)</td>
<td>0.23±0.06</td>
<td>1.0±0.65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gestational weeks at the first trimester US</td>
<td>11.7±0.5</td>
<td>11.8±0.5</td>
<td>0.086</td>
</tr>
<tr>
<td>NT (mm)</td>
<td>1.26±1.0</td>
<td>1.08±0.5</td>
<td>0.072</td>
</tr>
<tr>
<td>NT ≥3.0 mm</td>
<td>42 (4.4)</td>
<td>1 (0.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hCG-β (MoM)</td>
<td>0.91±0.88</td>
<td>1.03±0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Combined FTS risk ≥1/250</td>
<td>184 (19.1)</td>
<td>23 (2.4)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD or number (%).
PAPP-A= pregnancy associated plasma protein A, MoM= multiple of median,
NT=nuchal translucency, hCG-β=human chorionic gonadotropin-β, FTS=first trimester screening

Altogether, an adverse outcome was detected in 42.7% of pregnancies in the low PAPP-A group, compared to 9.5% in the group with normal PAPP-A levels (Figure 12).
In the group with low PAPP-A, a significantly lower incidence of pregnancies proceeding to delivery was detected. The incidence of aneuploidies and SA was significantly higher, but the risk of major structural anomalies was not increased (Table 10).
In the group with low PAPP-A, 86.1% (n=827) of pregnancies proceeded to delivery, compared to 99.1% (n=952) in the control group (p<0.001). The gestational age at delivery was significantly lower in the group with low PAPP-A. An increased incidence of PTD, PIH and PE was observed in the low PAPP-A group, as well as a significantly lower birth weight and significantly higher incidence of SGA, SB and emergency CS (Table 11).
Table 11. Pregnancy outcomes in study and control groups

<table>
<thead>
<tr>
<th>Outcome</th>
<th>PAPP-A &lt; 0.3 MoM (n=827)</th>
<th>PAPP-A 0.9-1.1 MoM (n=952)</th>
<th>p-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delivery with fetal abnormality*</td>
<td>15 (1.8)</td>
<td>1 (0.1)</td>
<td>&lt;0.001</td>
<td>17.4 (9.1-33.3)</td>
</tr>
<tr>
<td>Gw at delivery</td>
<td>39.1±2.6</td>
<td>39.9±1.7</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Gw at delivery, no fetal abnormality**</td>
<td>39.2±2.5</td>
<td>39.9±1.7</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Preterm delivery &lt;37 gw</td>
<td>111 (13.4)</td>
<td>48 (5.0)</td>
<td>&lt;0.001</td>
<td>2.5 (1.8-3.5)</td>
</tr>
<tr>
<td>Preterm delivery &lt;37 gw, fetal abnormalities excluded**</td>
<td>103 (12.7)</td>
<td>48 (5.0)</td>
<td>&lt;0.001</td>
<td>2.3 (1.6-3.3)</td>
</tr>
<tr>
<td>Preterm delivery &lt;34 gw</td>
<td>38 (4.6)</td>
<td>14 (1.5)</td>
<td>0.001</td>
<td>2.8 (1.5-5.2)</td>
</tr>
<tr>
<td>Preterm delivery &lt;34 gw, fetal abnormalities excluded**</td>
<td>33 (4.1)</td>
<td>14 (1.5)</td>
<td>0.005</td>
<td>2.5 (1.3-4.6)</td>
</tr>
<tr>
<td>Pregnancy induced hypertension</td>
<td>39 (4.7)</td>
<td>24 (2.5)</td>
<td>0.012</td>
<td>1.9 (1.1-3.2)</td>
</tr>
<tr>
<td>Pre-eclampsia</td>
<td>45 (5.4)</td>
<td>5 (0.5)</td>
<td>&lt;0.001</td>
<td>10.9 (4.3-27.6)</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>3121.9±720.5</td>
<td>3511.8±572.3</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Birthweight (g), fetal abnormalities excluded**</td>
<td>3142.7±697.3</td>
<td>3512.5±572.2</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Small for gestational age neonates</td>
<td>107 (12.9)</td>
<td>28 (2.9)</td>
<td>&lt;0.001</td>
<td>4.9 (3.2-7.5)</td>
</tr>
<tr>
<td>Small for gestational age, fetal abnormalities excluded**</td>
<td>100 (12.3)</td>
<td>28 (2.9)</td>
<td>&lt;0.001</td>
<td>4.9 (3.2-7.5)</td>
</tr>
<tr>
<td>Stillbirth***</td>
<td>9 (1.1)</td>
<td>0</td>
<td>&lt;0.001</td>
<td>n/a</td>
</tr>
<tr>
<td>Emergency Caesarean section</td>
<td>124 (15.2)</td>
<td>110 (11.6)</td>
<td>0.038</td>
<td>1.4 (1.0-1.8)</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD or number (%), PAPP-A = pregnancy associated plasma protein A, MOM = multiple of median, OR = odds ratio, gw = gestational weeks, n/a = not applicable, * deliveries with stillbirth chromosomal and structural anomalies; ** deliveries with stillbirth, chromosomal and structural anomalies excluded; *** chromosomal anomalies excluded.
The group with PAPP-A < 0.3 MoM was further divided into three subgroups according to the level of low PAPP-A. The incidence of different PAPP-A values increased with increasing level of PAPP-A, and the lowest PAPP-A values (<0.1 MoM; group 1) constituted only 3.1% of the group. The incidences of PAPP-A levels 0.1-0.2 MoM (group 2) and 0.2-0.3 MoM (group 3) were 21.0% and 75.9% (Figure 11).

**Figure 11.** The distribution of PAPP-A levels in three subgroups

The mean PAPP-A levels in the three subgroups were significantly different from each other (group 1 vs group 2, *p*=0.05). The mean maternal age, the body mass index and the incidence of primiparity and smoking were similar across the subgroups. In group 1, the first trimester US was done earlier and mean NT in groups 1 and 2 was larger than in group 3 (Table 12).
Table 12. Characteristics of the three PAPP-A subgroups

<table>
<thead>
<tr>
<th></th>
<th>Group 1 PAPP-A &lt;0.1 MoM (n=30)</th>
<th>Group 2 PAPP-A 0.1-0.2 MoM (n=202)</th>
<th>Group 3 PAPP-A 0.2-0.3 MoM (n=729)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAPP-A (MoM)</td>
<td>0.07±0.02</td>
<td>0.16±0.03</td>
<td>0.26±0.03</td>
<td>&lt;0.001&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maternal age, years</td>
<td>32.3±7.9</td>
<td>31.4±5.5</td>
<td>30.4±5.2</td>
<td>0.029&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)*</td>
<td>24.3±3.0</td>
<td>24.5±4.3</td>
<td>24.7±5.5</td>
<td>0.900</td>
</tr>
<tr>
<td>Primiparity</td>
<td>11 (36.7)</td>
<td>74 (36.6)</td>
<td>272 (37.3)</td>
<td>0.983</td>
</tr>
<tr>
<td>Smoking **</td>
<td>1 (3.3)***</td>
<td>19 (9.4)***</td>
<td>138 (18.9)***</td>
<td>0.017&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gestational weeks at</td>
<td>first trimester US</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT (mm)</td>
<td>2.0±1.9</td>
<td>1.5±1.4</td>
<td>1.1±0.8</td>
<td>&lt;0.001&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD or number (%)

PAPP-A= pregnancy associated plasma protein A, MOM=multiple of median, US=ultrasound

Dunn’s post-hoc test: a=significant difference between groups 1 and 2, b=significant difference between groups 1 and 3, c=significant difference between groups 2 and 3, d=Dunn’s post-hoc analysis did not show significant differences between groups

*missing: PAPP-A <0.1MoM: n=11 (37%); PAPP-A 0.1-0.2MoM: n=26 (12.9%); PAPP-A 0.2-0.3 MoM: n=6 (8.4%)
**missing: PAPP-A <0.1MoM: n=14 (46.7%); PAPP-A 0.1-0.2MoM: n=35 (17.3%); PAPP-A 0.2-0.3 MoM: n= 36 (4.9%)***expressed as percentage out of all pregnancies in the group

Many pregnancy outcomes were related to the level of PAPP-A. The risk of aneuploidy and SA significantly increased with decreasing PAPP-A, but the risk of structural abnormality did not differ between groups (Table 13).
Table 13. Pregnancies not proceeding to delivery in the study and control groups

<table>
<thead>
<tr>
<th></th>
<th>Group 1 PAPP-A (n=30)</th>
<th>Group 2 PAPP-A (n=202)</th>
<th>OR (95% CI)</th>
<th>Group 3 PAPP-A (n=729)</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aneuploidy</td>
<td>12 (40.0)</td>
<td>47 (23.3)</td>
<td>2.2 (0.99-4.9)</td>
<td>45 (6.2)</td>
<td>10.13 (4.6-22.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Structural</td>
<td>0 (0)</td>
<td>2 (1.0)</td>
<td>n/a</td>
<td>2 (0.3)</td>
<td>n/a</td>
<td>0.353</td>
</tr>
<tr>
<td>abnormality</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>13 (43.3)</td>
<td>8 (4.0)</td>
<td>18.5 (6.8-51.0)</td>
<td>9 (1.2)</td>
<td>61.76 (23.0-162.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>abortion</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Values are expressed as number (%)
PAPP-A = pregnancy associated plasma protein A, MOM = multiple of median, CI = confidence interval, OR = odds ratio, n/a = not applicable

Adverse pregnancy outcomes were also related to the level of PAPP-A. The likelihood of a liveborn delivery was significantly higher in groups 2 and 3, and gestational age as well as birthweight increased with increasing PAPP-A levels. The risk of PTD and SGA was lowest in the group with the highest PAPP-A values and the risk of SB was related to the level of PAPP-A. No difference was detected in the incidence of PE and emergency CS (Tables 14 and 15).
<table>
<thead>
<tr>
<th>Group A (n=79)</th>
<th>Group B (n=202)</th>
<th>Group C (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2-0.3 MAM</td>
<td>0.1-0.2 MAM</td>
<td>0.1 MAM</td>
</tr>
<tr>
<td>PAPP-A vs Group 1</td>
<td>PAPP-A vs Group 2</td>
<td>OR (95% CI)</td>
</tr>
</tbody>
</table>

Table 4. Pregnancy and delivery characteristics in PAPP-A subgroups
Table 15. Pregnancy and delivery characteristics in three PAPP-A subgroups

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>OR (95% CI) group 2 vs group 1</th>
<th>Group 3</th>
<th>OR (95% CI) group 3 vs group 1</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAPP-A</td>
<td>PAPP-A</td>
<td>PAPP-A</td>
<td>PAPP-A</td>
<td>PAPP-A</td>
<td></td>
</tr>
<tr>
<td>&lt;0.1 MoM (n=30)</td>
<td>14.06</td>
<td>60.09</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>0.1-0.2 MoM (n=202)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2-0.3 MoM (n=729)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All deliveries

- All deliveries, fetal abnormalities excluded
- Gestational weeks at delivery
- Birthweight (g)

values are expressed as mean±SD or number (%), PAPP-A= pregnancy associated plasma protein A, MOM=multiple of median, CI=confidence interval, OR=odds ratio, g=gestational weeks, *Kruskal-Wallis or χ² test, **chromosomal abnormalities excluded

a=significant differences between groups 1 and 2, b= significant differences between groups 1 and 3, c= significant differences between groups 2 and 3, d= no significant differences between groups, e= percentage of all deliveries in the group, f= percentage of liveborn deliveries
DISCUSSION

The significance of second trimester soft markers after normal FTS

Study I demonstrated four fetuses with a chromosome abnormality; one case of trisomy 18, one case of triploidy, a case of 21-chromosome mosaicism and one case with an inversion of chromosome 10. Of these, the first two represent severe chromosome abnormalities, that are always associated with a severe clinical condition. Both of these fetuses also had a severe growth restriction and small but morphologically normal placentas. At the combined FTS, these pregnancies had DS risks of 1/270 and 1/430, respectively, that would have been considered an intermediate risk, if screened using the contingent screening protocol.

In contrast to this, the inversion of chromosome 10 is a structural rearrangement of the chromosome, which has no effect on the phenotype of its carrier. An inversion of a chromosome occurs when a piece of chromosome breaks out and turns around 180 degrees. It may (pericentric) or may not (paracentric) include the centromere of the chromosome. Inversions are the common karyotype aberrations (Entesarian et al. 2009) and the inversion detected in the study, inv(10)(p11.2q21), is reported to be a phenotypically “silent” inversion with a frequency of 1/3600-1/12 800 in northern Europe (Entesarian et al. 2009).

Trisomy 21-mosaicism, on the other hand, is of potential clinical significance. Mosaicism is a condition in which an individual has two or more genetically distinct cell lines originating from a single zygote. In the case of trisomy 21 mosaicism, both euploid and trisomic cell lines exist and the risk of phenotypic abnormality increases with the increasing proportion of trisomic cells. DS is the most common chromosomal abnormality seen in liveborns, occurring at a frequency of 1/700-1/800 live births. It is of note, that the study by Wallerstein et al. (2000) showed a 50% risk of abnormal pregnancy outcome even in pregnancies with a 3-10% level of mosaicism in amniotic fluid. On the other hand, the phenotypic appearance of low level mosaicism may be subtle and thus, the condition unrecognized (Papavassiliou et al. 2014).

Thus, the overall incidence of diagnosed chromosome abnormalities was 4/158 (2.5%), but the outcome of pregnancies with the inversion of chromosome 10 and trisomy 21-mosaicism was a healthy newborn. Assuming that the chromosomes of these healthy newborns are normal, the
The incidence of clear-cut clinically relevant abnormalities is 2/228 (0.9%). However, we found three potentially relevant abnormalities and performed karyotyping in 158 cases (1.9%). Thus, one can speculate, that the incidence of clinically relevant abnormalities in the study was 0.9-1.9%.

After counseling, 30.3% of mothers declined AC for karyotyping. After the beginning of 2015, NIPT has been offered in our clinical practice in cases where two or more soft markers are present, and the uptake has been approximately 70-80%.

Soft markers were brought into screening as markers of DS (Benacerraf 2010a) in the 1980s. However, their significance is still considered to be controversial. Several things need to be considered when evaluating and comparing studies concerning the use of soft markers in second trimester DS screening.

The primary screening methods for DS are the first trimester NT and, especially, the combined FTS screening. They have DR of 76.8% (Nicolaides 2004) - 90% (Kagan 2008) and the uptake of combined FTS is high. This puts high requirements on the screening method used in the second trimester.

The majority of studies concerning the second trimester soft markers have been performed in high-risk populations with advanced maternal age, abnormal or no previous serum screenings (Cicero et al. 2003, Vintzileos et al. 1995, Wax et al. 2000). The results from these studies cannot be applied to pregnancies with normal FTS. Studies concerning the significance of soft markers after normal combined FTS are few (Aagard-Tillery et al. 2009, Krantz et al. 2007, Sood et al. 2010) and their results conflicting. Thus, the results of these studies have to be evaluated with respect to previous screenings of the study population.

The soft marker screening protocol is not standardized (Lau and Evas 2008). A wide variety of different protocols and definitions of individual soft markers are used: hence, the confusion. Neither is there any conclusion of the number or combination of soft markers to be used nor the method to integrate the information with the possible FTS result.

Several authors suggest that soft markers should be interpreted with respect to a priori risk, and interpreted differently if detected in isolation or combination (Bradley et al. 2005, Bromley et
al. 1997, Bromley et al. 2002b, Nyberg et al. 2001). Most authors also agree to modifying the a priori risk after the second trimester US. The new risk is assessed according to the presence or absence of soft markers, using the LR assigned to each soft marker (Agathokleous et al. 2013, Nyberg et al. 2001a, Raniga et al. 2006). Even risk reduction is used (Agathokleous et al. 2013), but is not advised by all (Lau, Evans 2008). At HUH, the uptake of the combined FTS is high. The a priori risk is not modified and soft markers are interpreted similarly with and without prior FTS. It is worth noting that if the risk is modified, all centers should have their own LRs based on their screening population, the FTS used, the methodology and the definitions of soft markers.

There are no guidelines concerning pregnancies with isolated soft markers. Of note, isolated markers are detected in 11-17% of euploid fetuses, but the prevalence is higher in DS (Nyberg et al. 2001a). Some authors are in favor of re-evaluating the risk, while others consider isolated soft markers to be non-significant. Isolated NF, ARSA and absent or hypoplastic NB are exceptions and should be considered significant even if detected in isolation (Ugurlucan et al. 2012).

Soft marker screening has certain limitations. It should be performed at the time of the second trimester US (18-22 gw). Earlier in gestation, the detection of these minor markers is uncertain and later, some markers are known to disappear (Picklemsimer et al. 2005) However, some studies have evaluated soft markers as early as 14 gw (Aagaard-Tillery et al. 2009, Renna et al. 2013, Viora et al. 2001), and some as late as the third trimester. In our institution, soft markers are used in screening only at the second trimester US.

The quality of the US equipment and the experience of the provider also affects the screening result. False negative and false positive findings are not uncommon, and this risk has to be recognized. In the study by Papageorghiou et al. (2008) and Vermeer et al. (2013), 13-15% of fetuses with SFs showed a normal femur length at follow-up scans. Maternal weight is also known to affect the screening result. Obese women are underscreened and the detection of soft markers decreases as the weight of the mother increases (Tsai, Ho et al. 2010).

The incidence of some soft markers varies according to race. EIF is detected in approximately 30% of euploid Asian fetuses, which is higher than the incidence among Caucasian fetuses with DS. EIF cannot, therefore, be used accurately to predict DS in Asian populations (Borgida et
al. 2005, Rebarber et al. 2004, Shipp et al. 2000, Shipp and Benacerraf 2002). Also, significant differences exist in the mean length of fetal femur. The fetuses of Asian or Hispanic mothers have been shown to be shorter compared to Caucasian and black mothers (Kovac et al. 2002, Shipp et al. 2001), and ethnic-specific charts should be used to avoid false positive findings in these populations (Kovac et al. 2002). Constitutional maternal factors also affect the growth of the fetus. Caucasian mothers shorter than -1 SD below the mean have been shown to have an increased fetal risk (RR 2.38) for SFs (BPD:FL) than mothers +1 SD above the mean (Drooger et al. 2005).

Opinions concerning the follow-up after detection of soft markers are conflicting. Due to the association with chromosomal abnormalities, surveillance after soft marker detection is advised by some authors (Bradley et al. 2005, Bronsteen et al. 2004, Filkins and Koos 2005, Leonardi et al. 1998, Sepulveda et al. 1995). This results in several rescreening appointments and may increase the negative emotions of mothers (Filly 2000, Getz and Kirkengen 2003). Surveillance is, however, not advised by all authors, and it is not systematically advised in our institution, whether or not the fetal karyotype is prenatally known.

*Genetic sonogram after the FTS*

The role of genetic sonogram and the use of soft markers after FTS is controversial. The study by Aagaard-Tillery et al. (2009) evaluated the effectiveness of second trimester US in modifying the maternal age-specific risk and the screening test risk previously set by different screening protocols. They found genetic US useful after combined FTS and second trimester quadruple test (DR 90%, FPR 5%) and a more modest effect after sequential protocols (DR 97-98%; FPR 5%). By using individual soft marker LRs, a simulation study by Kratz et al. (2007) described a 6.1% increase in DR for combined FTS-negative patients with an additional 1.2% in FPR (DR 94.6%, FPR 5.4%) and a 4.8% increase in DR with an additional 0.7% in FPR (DR 93.3%, FPR 4.9%) after contingent protocol (genetic US after combined FTS risk 1/300-1/2500).

A study by Sood et al. (2010) using individual positive and negative LRs (Nyberg et al. 2001b) is questioning the usefulness of soft markers in patients with low risk after combined FTS, and
the reassessment of DS risk based on second trimester US is clearly opposed by some authors (Lau and Evans 2008).

**Adverse pregnancy outcomes in euploid pregnancies with SFLB**

Our study demonstrated that euploid pregnancies with SFLBs had a significantly increased risk of adverse outcome regarding PTD, SGA, and SB, and the OR of any adverse pregnancy outcome was 24.9. The risk we saw is higher than most studies concerning SFLB, and may be explained by the definition of SFLB (< 3rd centile) used in our study and the inclusion of pregnancies with both SF and SH.

A plethora of SF and SH definitions is used in the existing literature. Compared to the 5th centile cut-off used in many studies (Papageorghiou et al. 2008, Ventura et al. 2012), our own cut-off limit may decrease the sensitivity for adverse outcomes. However, it reduces the FPR and, as a consequence, possible invasive procedures for assessing the fetal karyotype. It is noteworthy that different reference charts in different populations have been used in the literature, and most studies include pregnancies with SF only (Goetzinger et al. 2012, Mathiesen et al. 2014, Todros et al. 2004, Ventura et al. 2012, Vermeer and Bekker 2013, Weisz et al. 2008, Özlu and Ozcan 2013). This hampers the comparison of different studies and their results. Only a few smaller studies with different cut-off limits (<5th centile, < -1.5 SD) have evaluated pregnancies with both SF and SH (Fukada et al. 1997, de Carvalho et al. 2013). Therefore, mostly studies with SF only have to be used as a reference for our study, making objective evaluation of our results difficult.

In our study, 76% of the fetuses were delivered preterm. The proportion of spontaneous prematurity was fairly low (4.5%) and close to the risk in the Finnish population (Autti-Rämö et al. 2005). In the majority of cases, PTD was induced due to maternal or fetal distress.

In some studies, PTD has been shown to be independent of FGR, which is usually a sign of placental dysfunction (Goetzinger et al. 2012). Our findings concerning the risk of PTD are consistent with earlier studies (Goetzinger et al. 2012, Mathiesen et al. 2014, Ozlu and Ozcan 2013) and it has been assumed that SF is an early sign of abnormal placentation and placental dysfunction. Hence, it seems to have a role in the pathogenesis of preterm birth (Kim et al.
2002, Kim et al. 2003). However, conflicting results concerning SFLBs and PTD have also been shown (Weisz et al. 2008).

We did not detect any cases of skeletal dysplasia. In these pregnancies, the bone morphology is known to be abnormal and additional structural defects very common.

**The clinical significance of pregnancies with extremely low PAPP-A in the combined FTS**

In our study, we showed that low first trimester PAPP-A increases the risk of aneuploidies, SA, PTD, PE, SGA and SB.

Approximately 3% of all study group pregnancies with PAPP-A ≤0.3 MoM ended in SA. Compared to pregnancies with an optimal PAPP-A level (0.9-1.1 MoM), the risk of SA was significantly increased (OR 7.7). The risk of SA was inversely correlated to the level of PAPP-A, and the incidence among pregnancies with PAPP-A < 0.1 MoM was 43.3%.

These results are consistent with previous studies (Barrett et al. 2008, Dugoff et al. 2004, Goetzl et al. 2004, Kabili et al. 2004, Kwik and Morris 2003, Ong et al. 2000, Smith et al. 2002, Yaron et al. 2002) showing ORs of 2.5-14.53 with cut-off levels 0.25-0.5 MoM or <5th centile (Barrett et al. 2008, Brameld et al. 2008, Dugoff et al. 2004, Ong et al. 2000, van Ravenswaaij et al. 2011, Yaron et al. 2002). It is worth noting that the determinations for SA or pregnancy loss in previous studies are variable. They vary from a pregnancy loss <13 gw (Valbuena et al. 2015) to a pregnancy loss <20 gw (Barrett et al. 2008, Goetzl et al. 2004, Gupta et al. 2015) and up to <24 gw (Dugoff et al. 2004). Also, pregnancies with aneuploidy and/or structural anomaly were not systematically excluded from the study populations with SA. Since they are both independent and strong risk factors for SA, these pregnancies were excluded when evaluating the risk of SA in our study population. The value-related risk of pregnancy loss < 24 gw was evaluated in a large multicenter study by Dugoff et al. (2004) and the OR increased from 1.95 to 5.22 as the level of PAPP-A decreased from <10th centile (0.42 MoM) to <1st centile (0.28 MoM). In our study, the OR for SA with the lowest PAPP-A values (<0.1 MoM) was 61.8, compared to PAPP-A levels 0.2-0.3 MoM.
In our study, the risk of nonchromosomal structural anomalies was not increased in pregnancies with low PAPP-A. The result is consistent with most previous studies, although a few conflicting results have also been reported (Barret et al. 2008).

Due to the high incidence of aneuploidies and SAs, the number of pregnancies proceeding to delivery was significantly lower in the group with low PAPP-A. The likelihood of delivery was very low in the group with the lowest (16.7%) PAPP-A values (<0.1 MoM), but increased to 92.3% in the group with PAPP-A 0.2-0.3 MoM. Also, the gw at delivery increased with increasing PAPP-A value.

In our studies, the risk of PTD was significantly increased among pregnancies with low PAPP-A and a trend of an increasing incidence with decreasing PAPP-A was detected. The risk remained significant when excluding pregnancies with fetal abnormalities, indicating the independent role of low PAPP-A. Previous results concerning the association of low PAPP-A with PTD are conflicting. An increased risk of PTD has been shown in many studies (Barrett et al. 2008, Kabili et al. 2004, Krantz et al. 2004, Ong et al. 2000, Smith et al. 2002, Spencer et al. 2005), but some authors dispute this association (Canini et al. 2008, Goetzinger et al. 2010, Kirkegaard et al. 2010, Kwik and Morris 2003, Morssink et al. 1998, Saruhan et al. 2012, van Ravenswaaij et al. 2011, Yaron et al. 2002). Also, the study population sizes and cut-off levels in previous studies are variable, hampering conclusions concerning the risk of PTD.

An important consideration is the etiology of PTD. Many studies report associations with PTD, but few point out the exact etiology responsible for PTD. Since low level of PAPP-A is an indicator of poor placental implantation and function, many PTDs are, in fact, induced deliveries due to other adverse outcomes, such as PE, SGA or SB. In the study by Kirkegaard et al. (2010), the risk of PTD decreased when the determination was restricted to spontaneous PTDs only. Our studies included all pregnancies with PTD, thus, they simply describe the risk of being born premature.

In our study, a slightly increased risk of PIH was detected in the group with low PAPP-A (OR 1.9). Most previous studies have concentrated on evaluating the risk of PE and the association with PIH is less studied. We also found the incidence of PE to be significantly higher among
pregnancies with low PAPP-A compared to pregnancies with optimal PAPP-A, the OR being 10.9.

The increased risk of PE is described in many other studies (Ay et al. 2005, Krantz et al. 2004, Odibo et al. 2011, Ong et al. 2000, Poon et al. 2009, Smith et al. 2002b, Spencer et al. 2005, Yaron et al. 2002), and a stronger association of low PAPP-A with early-onset PE has been suspected (Poon et al. 2009, Poon and Nicolaides 2014, Sharp and Alfrevic 2014). However, some studies do not agree with an increased risk of PE (Ay et al. 2005, Canini et al. 2008, van Ravenswaaij et al. 2011, Saruhan et al. 2012, Tul et al. 2003) and high levels of PAPP-A have also been detected in pregnancies with PE (Bersinger et al. 2003, Bersinger and Odegard 2004, Deveci et al. 2009). Different cut-offs also hamper the comparison of results (Gupta et al. 2015, Ranta et al. 2011, Yaron et al. 2002).

The association of low PAPP-A and PE can be explained by the underlying pathophysiology. Poor invasion of extravillous trophoblasts to the endometrium and maternal arteries was already described in the 1990s (Meekins et al. 1994). This leads to retention of vasoconstrictive capacity and stiffness of the vessels later in pregnancy (Smith, Dunk et al. 2009). Low maternal PAPP-A is a reflection of placental dysfunction already in the first trimester, but the high BP and multiorgan dysfunction only becomes detectable in the second or third trimester of pregnancy.

The cut-off limits used in previous studies are variable (Gupta, Goyal et al. 2015, Ranta, Raatikainen et al. 2011, Yaron, Ochshorn et al. 2002). Due to a very small number of deliveries in the group with the lowest PAPP-A values, we were not able to show a PAPP-A value-related risk of PE. Based on the etiology of PE and the association with placental function, one may assume that a value-related risk could be shown in a larger cohort. In our study, only 16.7% of pregnancies with PAPP-A <0.1 MoM proceeded to delivery, addressing a need for a substantially larger cohort to reach statistical significance in outcomes concerning this subgroup.

The birthweight of fetuses in pregnancies with low PAPP-A was significantly lower compared to pregnancies with an optimal level of PAPP-A. Birthweight also appeared to be value related and increased with increasing PAPP-A values. The same result is reported by Habayeb et al (2010).
The birthweight of fetuses in pregnancies with low PAPP-A was significantly lower compared to pregnancies with an optimal PAPP-A value. Birthweight also appeared to be value related and increased with increasing PAPP-A values. The same result is reported by Habayeb et al (2010).

Impaired fetal growth is not yet detectable in the first trimester, but in the second and third trimester, reduced fetal growth has been reported (Fox and Chasen 2009). It is unclear at what point the restricted growth becomes detectable, but it has been speculated to happen late in the first trimester, when a switch from histiotropic to haematotrophic placentation occurs and can be detected as maternal and fetal Doppler changes (Jauniaux et al. 1992).

Consistent with other studies (Barrett et al. 2008, Carbone et al. 2012, Cowans and Spencer 2007, Dugoff et al. 2004, Kabili et al. 2004, Krantz et al. 2004, Kwik and Morris 2003, Marttala et al. 2010, Ong et al. 2000, Peterson and Simhan 2008, Spencer et al. 2005, Spencer et al. 2008, Yaron et al. 2002), our study showed a significantly increased risk of SGA in pregnancies with low PAPP-A. Compared to pregnancies with optimal PAPP-A, the OR in the low PAPP-A group was 4.9. It is again worth noting that different determinations for low PAPP-A, as well as impaired fetal growth, have been used in previous studies, complicating the evaluation of poor fetal growth associated with low PAPP-A. The overlap of true growth restriction and constitutional smallness and the impact of other placenta-related complications is difficult to exclude. Previously, some conflicting opinions concerning the association of low PAPP-A and SGA have also been reported (Morssink et al. 1998).

We detected nine cases of SB in the group with low PAPP-A, compared to none in the control group. Based on the statistics, the incidence of SB in the pregnant Finnish population is 0.3%, therefore, the risk for SB in pregnancies with low PAPP-A can be considered increased. Another Finnish study using the same determination for low PAPP-A (≤0.3 MoM) reported an increased risk with OR 3.59 (Marttala, Peuhkurinen et al. 2010). However, all studies do not confirm the association of low PAPP-A and the risk of SB (Yaron, Heifetz et al. 2002, RW.ERROR - Unable to find reference:791)

The mode of delivery is studied by few authors. We detected a minor increase of CSs in the group with low PAPP-A, but due to the small number of cases, could not detect a difference related to the level of PAPP-A. A previous study by Gupta et al. (2015) also reported an increase in the risk of CS.
We did not study the association of PAPP-A level with placental abruption, but previous studies have shown conflicting results (Gupta, Goyal et al. 2015, Ranta, Raatikainen et al. 2011, Brameld, Dickinson et al. 2008). Additionally, studies concerning the association with placenta previa (Brameld et al. 2008, Wang et al. 2014) and gestational diabetes are ambiguous (Dugoff et al. 2004, Ong et al. 2000, Smith et al. 2002b).

**Low PAPP-A and screening of adverse pregnancy outcome**

In conclusion, the association of low PAPP-A with adverse outcome has been shown in numerous studies. Unfortunately though, low PAPP-A does not provide a screening tool for poor pregnancy outcome (Brameld, Dickinson et al. 2008). In a study by Barrett et al (2008), the sensitivity with a cut off-value 0.3 MoM was 5.1% with a 10.0% FPR. The sensitivity of low PAPP-A for impaired fetal growth also remains low in previous studies and is reported to be 15-33% with a 20% FPR (D'Antonio et al. 2013, Carbone et al. 2012). The DR remains poor even when combining the PAPP-A result with 2D assessment of fetal growth (Carbone et al. 2012).

Low PAPP-A alone has poor sensitivity for PE. However, it has been suggested that first trimester UtaD could be effective in screening for PE, and DRs of 47.8% and 26.4% with FPRs of 7.9% and 6.6% have been reported for early and all PE, respectively (Velauthar et al. 2014). The combination of UtaD with maternal factors leads to an even higher DR (81%, 0% FPR) compared to maternal history only (47%) when screening for PE requiring delivery < 34 gw (Poon, Staboulidou et al. 2009). Maternal arterial stiffness, assessed by increased systolic BP in the first trimester, can also be used as a marker for PE. It has some predictive capacity, especially when combined with maternal characteristics, and can achieve a DR of 56.9% (10% FPR) (Khalil, Akolekar et al. 2012).

The association of low PAPP-A and PE is explained by the underlying placental dysfunction, reflected by reduced production of PAPP-A (Peterson and Simhan 2008, Spencer et al. 2008a). Despite this association, low PAPP-A is not useful in screening for PE, and only 8-23% of pregnancies with PE have been shown to have PAPP-A values <5th centile (~0.4 MoM). In previous studies concerning the association with PE, the reported ORs have been 1.5-4.6 (Dugoff et al. 2004, Ong et al. 2000, Pilalis et al. 2007, Poon and Nicolaides 2014, Smith et al. 2002b, Spencer et al. 2005, Spencer et al. 2007, Yaron et al. 2002). At a 5% and 10% FPR, the DRs for PE requiring delivery <34 gw were 44% and 55% and for PE requiring delivery <37 gw 37% and 48%, respectively. When adding the assessment of mean arterial pressure and
uterine artery PI, the DRs with 5% and 10% FPR increased to 82% and 93% and to 53% and 75%, respectively (Poon and Nicolaides 2014).

The 2D-US for placental surface area combined with first trimester PAPP-A is reported to be of increased value (Suri, Muttukrishna et al. 2013), and placental bed vascularity (<10th centile), assessed by 3D-power-Doppler, has been reported to have DRs of 51% for PE and up to 60% for PE requiring delivery before 34 gw. The DR for PIH was only 30% (10% FPR) (Hafner, Metzenbauer et al. 2013) However, the skill required for this measurement is likely to inhibit the use of this technique.

In a clinical setting, the combination of maternal factors, UtaD, BP and PAPP-A has been demonstrated to achieve a DR of 80% for PE with 10% FPR (Scazzocchio et al. 2013), while other studies have been less optimistic, reporting a 60% DR with a 20% FPR (Di Lorenzo et al. 2012).

Our study also showed an increased risk of PTD, and lower levels of PAPP-A have been detected in women with subsequent spontaneous PTD. However, PAPP-A is not superior to maternal characteristics as a screening tool for PTD (Barrett et al. 2008, Beta et al. 2011).

Even though we detected a significantly increased number of SBs in the group with low PAPP-A, the sensitivity of low PAPP-A alone as a screening test for SB is too poor for clinical use (Barrett et al. 2008, Dugoff et al. 2004, Karim and Sau 2013, Kwik and Morris 2003, Marttala et al. 2010, Spencer et al. 2006, Yaron et al. 2002). Low PAPP-A together with chronic hypertension is also associated with subsequent SB (Akolekar et al. 2011), but neither shows potential predictive value for use in clinical practice (Akolekar et al. 2011). In one study, low levels of first trimester PAPP-A (<0.4 MoM) (Smith et al. 2004) were found to have high accuracy for SB related to PA or SGA (LR+ 14.1, LR- 0.3), especially SB before 33 gw. The accuracy for SB not related to these complications was low. Also, a high (>90th centile) uterine artery PI has been shown to have moderate to high predictive accuracy for SB related to PA, SGA or PE before 32 gw (LR+ 8.0-10.0, LR– 0.0-0.2 ), but a low predictive value for all-cause SB >32 gw (Poon et al. 2013). Another study evaluating the combination of PAPP-A <0.8 MoM and low soluble luteinizing hormone (LH)/hCG receptor (sLCGR) showed a DR of 52.3% (FPR 7.1%) for SB in euploid fetuses (Chambers et al. 2012).
SB is characterized by multiple different etiologies (Di Renzo 2009, Romero 2009), which can explain the poor performance of tests to predict all-cause SB. DRs for late SB ( >24 gw) based on maternal characteristics and biomarkers are too poor to be used in clinical practice (Dugoff et al. 2008). A combination of low PAPP-A together with high UtaD PI might, in theory, have a good predictive value based on the ability to reflect both poor placental function and perfusion.

As is true in the screening of any adverse outcome or disease, an intervention should be available when introducing a new screening method. Even though the sensitivity of low PAPP-A can be increased by combining with other screening markers of adverse outcome, the main treatment remains the timing of delivery.

**Surveillance of pregnancies with low PAPP-A**

Due to adverse outcomes associated with low PAPP-A, some authors have concluded that pregnancies with low first trimester PAPP-A warrant more intensive surveillance. They are in favor of growth scans, UtaD measurements and cervical length measurement at 22 gw (Barrett, Bower et al. 2008, Spencer, Yu et al. 2005, Kwik, Morris 2003). However, no consensus exists on the frequency and principles of screening. The need for surveillance is not uniformly supported (Ay et al. 2005, Canini et al. 2008, Dugoff et al. 2004, Goetzl et al. 2004, Huynh et al. 2014), especially concerning surveillance in otherwise normal pregnancies (Brameld et al. 2008, van Ravenswaaij et al. 2011) and the lack of effective interventions (Brameld et al. 2008, Lindqvist and Molin 2005).

**Prenatal screening and anxiety**

Technological advances have brought us the possibility of detecting minor structural details with uncertain value (Filly 2000, Getz and Kirkengen 2003). Together with the varying methodology, it brings challenges in counseling patients and gives rise to strong emotional and social implications.
US is a positively anticipated event for parents, and they are poorly prepared for uncertain or bad news (Eurenius et al. 1997, Filly and Crane 2002, Getz and Kirkengen 2003, Lalor and Begley 2006, Santalahti et al. 1998, Sommerseth and Sundby 2010, Watson et al. 2002). Information about soft markers rises feelings of shock, disbelief and distress (Filly 2000, Watson et al 2002, Åhman et al. 2010), and many patients have not heard about them before the screening (Cristofalo et al. 2006). Some are left not understanding. (DiPietro et al. 2006), with this diagnosis of uncertain value. In one study, the minority (2/11) of women stated that they wanted to be informed about the detection of soft markers, while others were hesitant to say they would rather not have known (Åhman et al. 2010). For some women, knowledge is not a benefit, but rather seen as a drawback, bringing anxiety: “Knowing is not always best” (Kohut et al. 2002).

These ambiguous emotions may be more difficult to handle than a diagnosis of true abnormality (Sparling et al. 1988), and if the possibility of “being at risk” was not considered before screening, the anxiety may be even stronger (Weinans et al. 2000). Womens’ preparedness and the ability to receive information in a stressful situation is limited, and sometimes the time window for decisions concerning karyotyping is short (Sommerseth and Sundby 2010, Åhman et al. 2010).

Accurate information, privacy and empathy are considered very important, and lack of information or inaccurate information very frustrating (Sayakhot and Carolan-Olah 2016). After counseling by the US provider, many women seek additional information and sometimes believe that the information they found themselves was better than that given during the US (Cristofalo et al. 2006). Despite counseling, the information received from friends and relatives is considered important (Åhman et al. 2010) and after screening, women feel that sufficient prescreening information would have decreased anxiety (van den Berg et al. 2008, Åhman et al. 2010).

In some studies, anxiety was reduced after counseling (Watson et al 2002), while some report that even low risk women cannot feel reassured after counseling (DiPietro et al. 2006). Different maternal self-protectiveness methods increase the risk of impaired mother-child attachment. At first, pregnancies are put “on hold” until delivery or final results (Åhman et al. 2010), and some reveal anxiety by distancing themselves from the fetus or “trying not to be pregnant” (Carolan and Nelson 2007). The anxiety may have long-lasting effects, and poor prenatal attachment to
the “possibly damaged” child (Åhman et al. 2010) is likely to postnatally effect the mother–child relationship (Benoit et al. 1997, Laplante et al. 2004, Mulder et al. 2002, Paarlberg et al. 1995). Also, the concern regarding soft markers often continues into the postpartum period, even though reassurance of the likely benign finding is given (Viaux-Savelon et al. 2012).

High quality prescreening information has to be a standard in prenatal maternal care. Information given by the referring providers has to be of good quality and equal to that given in the university hospital (DiPietro et al. 2006). Only with sufficient information, the principle of autonomy is met and womens’ decisions can be considered to be informed choices (O’Cathain et al. 2002, van den Berg et al. 2008, van den Heuvel et al. 2008)

The future of prenatal screening

In 1997, cell free fetal DNA (cffDNA) was first shown to be present in the plasma of pregnant women (Lo et al. 1997). This discovery was a technological breakthrough providing a possibility for non-invasive prenatal screening of fetal conditions. In 2011, this new method was introduced into clinical practice as a non-invasive prenatal test (NIPT) (Hartwig et al. 2017).

NIPT has shown to have superior performance compared to traditional FTS methods such as combined FTS and NT measurement. It is well evidenced for detecting trisomies 21, 18 and 13 and it can also detect sex chromosome aneuploidies. The sensitivity for DS has been found to be 99.2% and the specificity 99.91%. Also, the PPV is highest for DS (85%) and lowest for sex chromosome aneuploidies (38%). The test can be done from 10th gw (Gil et al. 2015) and there is no risk of test-related MC.

CffDNA in mainly derived from the cytotrophoblast of chorionic villi in placenta. This tissue is not always representative for the fetus and discrepancy between placental and fetal karyotype may exist. Thus, a false positive or a false negative result is possible and NIPT can not be considered as a diagnostic test. A positive NIPT result has to be confirmed by an invasive test, either CVS or AC (Hartwig et al. 2015).

The experiences concerning the discordant results of NIPT and invasive test are still few. A
recent study by Hartwig et al. (2015) found false positive results to be much more common than false negative results. Most of these false positive cases (67%) showed no obvious explanation for the discordant result, but placental and maternal mosaicism, maternal copy number variation, vanishing twin, maternal malignancy and technical or human errors was found to be possible reasons for discordance. The false negative results, including cases of NIPT indicating an abnormality different from the one present in the fetus, were mainly explained by true fetal mosaicism and some cases by a low fraction of cffDNA in maternal circulation. In few cases (0.88%) the result of NIPT can not be reported due to low fraction of cffDNA (0.59%), an uninformative DNA pattern or technical issue (0.29%) (Strom et al. 2017).

The development of array-based cytogenetic techniques has improved the possibilities to detect even smaller genomic abnormalities, so called copy-number variants, that are not routinely seen on karyotyping. This means genetic deletions or duplications, which results in a variation from the expected number of copies of a segment of DNA. Depending on their location and genetic content, these copy-number variants can be either benign or pathogenic. In pregnancies with a normal karyotype, microarray analysis has revealed clinically relevant deletions or duplications in 6% of the fetuses with structural anomaly and in 1.7% of the pregnancies with positive screening result or advanced maternal age (Wapner et al. 2012).

The accuracy as a screening method and decreasing expenses of NIPT will most probably broaden the availability and use of the test in the future. The technological development may also broaden the scope of prenatal screening. However, the role of combined FTS as a first line screening test has to be defended. The first trimester US has an essential role in evaluating the pregnancy viability, number of fetuses and gestational age in the early pregnancy. The information gained from the fetal NT and maternal serum markers is also valuable in assessing the risk of possible structural abnormalities and adverse outcomes other than aneuploidies.

Prenatal screening bears fundamental ethical questions. The genome-wide prenatal screening will soon be technically achievable without invasive methods. This attraction of the scientific possibilities holds major pitfalls and the danger of information overload, counseling difficulties and leaving many questions with no answers. The primary goal is to offer meaningful reproductive information and an informed consent should remain the target of counseling. ‘Primum non necere’, the golden standard of patient care, has to be re-evaluated again to meet

**Strengths of the study**

All pregnancies in studies I and II were considered low risk after FTS, which can be considered a strength of the study. Also, multiple pregnancies and those without FTS were excluded. Populations in this study were also ethnically homogenous and, different from some previous studies, fetal structural anomalies were excluded in studies I and II.

In study II, we also tried to exclude the influence of false positive findings of SF and SH. This has typically not been done in previous studies. Data on Doppler measurement and placental histology were also available in most study group pregnancies.

In comparison with other studies concerning very low PAPP-A, the size of the study and control groups in study III can be considered a strength. The baseline characteristics of groups were also comparable. While evaluating the risk of low PAPP-A, the study also demonstrated an excellent prognosis of pregnancies with PAPP-A level near 1 MoM. In study IV, we demonstrated the increasing risks associated with decreasing PAPP-A, which has rarely been seen in previous studies.

**Limitations of the study**

The retrospective study design can be considered a limitation of this study, as well as the lack of data on maternal comorbidities and medication in studies II-IV. The ethnic background of the patients was not analyzed, since it is not systematically collected. However, study populations were very homogenic, with a strong predominance of white patients.

In HUH, NF is not screened in the second trimester US, which is a limitation of study I. The significance of NF as a soft marker is, however, acknowledged and one can speculate as to whether or not it would have been recognized if detected during the US. Even though the
association of different soft markers with DS is variable, all patients received the same counseling concerning the risk of DS.

The small number of cases in studies I and II restricts the studies’ power. Larger study groups would provide more precise information concerning the significance of soft markers after normal FTS, as well as the significance of SF and SH in euploid fetuses during the second trimester of pregnancy.

Despite the good size of study and control groups in study III, the clinical context of the study results is limited to a small group of pregnant women. Studies III and IV lack statistical power concerning rare outcomes and, due to the small incidence of the lowest PAPP-A values, the size of subgroups in study IV is very variable.

One might criticize the selection of the control group in study III since a “gray zone” (PAPP-A 0.3-0.9) between study and control groups was not analyzed. This study design is, however, crucial to describe the significance of low PAPP-A compared to pregnancies completely lacking this risk factor. The evaluation of the gray zone and outcomes associated with decreasing PAPP-A values would, however, yield more information concerning the deterioration of pregnancy outcome.
CONCLUSIONS

On the basis of the present work, the following conclusions can be drawn:

1. After normal FTS, second trimester soft markers have low specificity for detecting DS. However, a chromosomal abnormality can still be found in 0.9-1.9% of pregnancies.

2. The risk of adverse pregnancy outcome is significantly increased in euploid, non-anomalous pregnancies with short femur and short humer in the second trimester US. Increased surveillance of these pregnancies is advisable.

3. Extremely low PAPP-A in the first trimester of pregnancy is associated with an increased risk of aneuploidy, spontaneous abortion and adverse pregnancy outcome. There is a trend of increasing risks with decreasing PAPP-A values. Even though low PAPP-A is a sign of increased risk, it is not useful as a screening tool.
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Marja Kaijomaa
APPENDIX

Fetal femur  Pyelectasis

Echogenic bowel  Echogenic intracardial focus

Choroid plexus cyst
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