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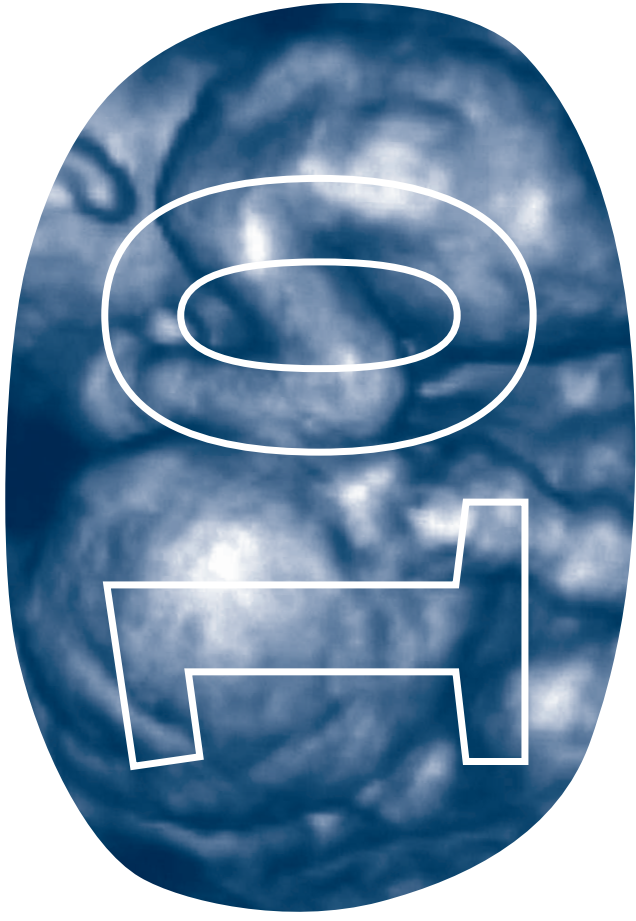
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## General discussion and perspectives

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The past decade prenatal screening for Down syndrome (DS) has developed from primarily second trimester serum screening towards first trimester combined screening. Nowadays prenatal screening is also shifting from screening for only fetal chromosomal anomalies towards combined screening for maternal health and adverse outcome of pregnancy.

For prenatal screening in twin pregnancies several issues have to be addressed: how to interpret serum marker values since they are pregnancy specific? Is screening in the second or the first trimester of pregnancy the most effective for twins? In case first trimester screening is advocated in twin pregnancies what kind of first trimester screening to perform: single nuchal translucency (NT) or first trimester combined screening? Using NT measurements, which method to choose; separate individual measurements leading to a fetal specific risk or the mean of two measurements, the largest or the smallest for calculating a pregnancy specific risk? Finally, should serum markers be corrected for twin chorionicity in first trimester combined screening? In this general discussion and the chapters of this thesis different issues concerning prenatal screening in twin pregnancies are described. Additionally, recent advances that are made in screening for adverse pregnancy outcome in twin pregnancies are addressed.

### PRENATAL SCREENING FOR TRISOMY 21

#### • • • Maternal age

The risk of DS affected pregnancy increases with maternal age<sup>12</sup>. This maternal age specific risk is the a priori or background risk used in the estimation of a screening test result. Generally in monozygotic twin pregnancies the assumed DS risk is identical to that of a singleton pregnancy<sup>34</sup>. This general assumption ignores the rare possibility of heterokaryotypic monozygotic twins, namely discordance for a chromosomal anomaly within a monozygotic twin pair<sup>56</sup>. In dizygotic twins the risk of at least one affected fetus is greater than expected from a singleton pregnancy<sup>34</sup>. However, according to Cuckle (1998) it is probably best to assume that the prior term risk for DS in twins does not differ from that of singletons<sup>7</sup>. Wald and colleagues in their 'pseudorisk' model also assume in dichorionic twin pregnancies the risk of each fetus being affected is on average half that in a singleton fetus<sup>8</sup>. However, it has been debated whether the age-specific risk should be applied per dichorionic fetus instead per dichorionic twin pregnancy. The above described theoretical birth rates of aneuploidy in twins do not corroborate completely with observational studies. A meta-analysis of 106 DS twin cases demonstrated a birth rate about 3% greater than for singletons<sup>7</sup>. In contrary Jamar et al. reported on a lower frequency of DS in a population of twin pregnancies as compared with the observed incidence in singleton pregnancies in the same period<sup>9</sup>.

#### • • • Serum screening

Serum marker concentrations are higher and on average doubled in twin pregnancies compared with singletons. Interpreting serum levels in twins is difficult since serum levels are pregnancy specific. In case of a discordant anomaly it is not possible to identify the proportion of the affected fetus and serum levels may be masked by the unaffected twin. In general, data are scarce on serum level distributions in discordant and concordant DS twins in both first and second trimester.

#### • • • Second trimester serum screening

Since 1984 studies have reported on low second trimester maternal serum alpha-fetoprotein (AFP) levels in DS singleton pregnancies<sup>10</sup>. Moreover altered levels of serum human chorionic gonadotropin (hCG) and its subunits were discovered in second trimester of aneuploid singleton pregnancies<sup>11</sup>. In 1988 study groups by Canick and Wald reported on reduced second trimester maternal serum unconjugated estriol (uE3) in DS singleton pregnancies<sup>12,13</sup>. In general AFP and uE3 levels are decreased and hCG increased in second trimester serum of DS singleton pregnancies. A meta-analysis of eight published studies on median second trimester DS markers in unaffected twin pregnancies reported an AFP of 2.26 (n=1314), uE3 1.68 (n=696), hCG 2.06 (n=890), and free  $\beta$ -hCG 2.07 (n=844)<sup>7</sup>. Only one study reported on second trimester inhibin A, a median of 1.99 in unaffected twin pregnancies (n=200)<sup>14</sup>. Muller et al. (2003) reported on second trimester maternal serum markers in 3043 twin pregnancies and found a median AFP of 2.10 and a median free  $\beta$ -hCG of 2.11. In this study the median AFP was not significantly different between mono- and dichorionic pregnancies. However, free  $\beta$ -hCG was significantly higher in monochorionic (2.16 MoM, n=245) compared with dichorionic (2.07 MoM, n=1317) twin pregnancies. Screen positive rate (SPR) and detection rates (DR) were better using second trimester AFP and free  $\beta$ -hCG (divided by factor 2) namely less than 8% and 55% respectively, versus screening based on maternal age alone (cut off 1:250)<sup>15</sup>. The largest study on second trimester serum screening includes 11,040 twin pregnancies with 27 DS cases. A DR of 71.0% in concordant DS twins and a DR 60.0% in discordant DS twins for an overall 10.8% false positive rate (FPR) were described compared with 74.4% DR and 10.3% FPR in singletons (cut-off 1:250)<sup>16</sup>.

#### • • • First trimester screening

In the 90-'s maternal biochemical markers for DS in the first trimester of pregnancy were studied. It turned out that first trimester free  $\beta$  subunit of hCG (free  $\beta$ -hCG) and pregnancy associated plasma protein-A (PAPP-A) combined with an ultrasound measurement of NT, are valuable markers to detect DS. In general first trimester screening allows earlier diagnosis of fetal anomalies than second trimester screening. In twin pregnancies first trimester screening is preferred over second trimester screening since the fetus specific NT measurement identifies the fetus at risk unlike pregnancy specific serum markers. Both first trimester single NT measurements and first trimester combined screening have better screening performances than second trimester serum screening in twin pregnancies.

### • • • First trimester Nuchal Translucency

Nuchal Translucency (NT) is the measurement of the thickness of a fluid collection in the neck of the fetus. NT measurements are commonly used as screening marker for aneuploidy between 11 and 14 weeks of gestation. Increased NT is not only associated with aneuploidy but also with a variety of structural fetal anomalies, mainly cardiac defects, genetic syndromes and adverse outcome of pregnancy<sup>17-19</sup>. Especially in higher order multiple pregnancies the NT is effective to identify the specific fetus at risk. The sensitivity of the NT measurement as risk estimation for DS in twin pregnancies is similar to singletons: 88% DR (7 of 8 DS cases) at with a FPR of 7.3%. However, the specificity is lower, since increased NT is more frequently seen in euploid MC twin fetuses (8.4%) compared with euploid dichorionic fetuses (5.4%)<sup>20</sup>. Using NT as a screening method for DS gives the opportunity to calculate fetus specific risks based on the individual measurements or a pregnancy specific risk based on the higher, the smaller or the average NT value. Wald and colleagues proposed to calculate a single risk estimate, thus pregnancy specific, with the use of the mean of two NT measurements in monozygotic twin pregnancies<sup>8</sup>. Vandercruys et al. reported on 769 MC twins undergoing NT screening, including 6 DS cases, using the average NT as an effective screening method. The estimated risk was 1:300 or more in 100% of the DS cases using the higher, 66.7% using the smaller and 100% using the average NT measurements. FPR was reduced using the average NT in euploid MC twins, namely 13.9% (cut-off 1:300) compared with 19.4% if the largest measurement was used<sup>21</sup>. Recently, Cuckle and Maymon published data on the between-fetus correlation coefficient of log NT in unaffected twin pregnancies, earlier described by Wojdemann et al.<sup>22,23</sup>. According to this study fetus specific DS risks in twin pregnancies should be calculated using its own NT value as well as that of the co-twin for both MC and DC twins, since there is a correlation between the measurements ( $r = 0.45$  ( $p < 0.0001$ ))<sup>22</sup>. In the near future risk estimations for DS in twin pregnancies should incorporate this between fetus correlation coefficient as a co-variable for NT measurements.

### • • • First trimester combined test

The last decade several studies reported on median values of first trimester serum markers in twin pregnancies. In general first trimester marker distributions of PAPP-A and free  $\beta$ -hCG are doubled in twins compared with singletons<sup>24-33</sup>. In a meta-analysis by Spencer the median free  $\beta$ -hCG MoM was 2.04 ( $n=825$ ) and the median PAPP-A MoM was 1.83 ( $n=707$ )<sup>34</sup>. Meta-analysis by Cuckle and colleagues reported on a median free  $\beta$ -hCG MoM of 2.07 and PAPP-A of 1.96<sup>35</sup>. Screening performance of the first trimester combined test in twins was published in a mathematical model described by Spencer in 2000 with a DR approaching 80%<sup>37</sup>. Prospective data from a case study and in further series with clinical data confirmed this Spencer 2000 model reporting a DR 75% (three out of four discordant DS cases) with 9.0% FPR per pregnancy and 6.9% per fetus<sup>27,28,36</sup>.

First trimester combined screening in twin pregnancies compared with single NT measurements reduces predominantly the FPR, from 14.3% towards 5.1% per pregnancy in a study of 100 consecutive twin cases including only three DS twin cases by Gonc et al.<sup>33</sup>. The most recent report on clinical performance of the first trimester screening in twin pregnancies shows a DR of 83.3% (7 DS cases) at 5% FPR with single NT screening

compared with a 100% DR if first trimester combined screening is used<sup>37</sup>.

Differences in marker distributions of free  $\beta$ -hCG and PAPP-A between mono- and dichorionic twin pregnancies were at first found not existing<sup>38</sup>. In the first models of first trimester combined screening in twin pregnancies no correction for chorionicity was thus applied<sup>38,39,38</sup>. Only recently Spencer et al. (2008) reported on more than 1914 twins, with 1214 cases of known chorionicity. They found a significantly lower PAPP-A in monochorionic twins compared with dichorionic, namely 1.76 MoM versus 2.25 MoM. For free  $\beta$ -hCG they found a tendency towards lower values in monochorionic twins<sup>39</sup>. In this thesis (Chapter 2) we found that biochemical markers in monochorionic twin pregnancies are significantly lower for both free  $\beta$ -hCG and PAPP-A<sup>40</sup>. We believe that a possible explanation for this finding is that in our study samples were taken earlier in pregnancy compared with the study by Spencer et al. (2008) in which samples were taken mainly after 12 weeks of gestation<sup>39</sup>.

Data on more than 5000 twin pregnancies presented at the Fetal Medicine Foundation Conference 2010 were in accordance to our findings and underline the necessity to correct first trimester serum levels for chorionicity. Moreover, in this study a gestational age specific increase in serum marker values for monochorionic and dichorionic relative to singletons was found<sup>41</sup>. The results of this large study on twin pregnancies must lead to further adaptation of the current screening algorithm programs. So far these algorithms were based on the earlier described meta-analysis by Spencer or Cuckle and colleagues using a twin-to-singleton correction factor<sup>34,35</sup>. Correction factors including chorionicity based on the study by Spencer et al. (2008), namely 2.023 for free  $\beta$ -hCG in twins compared with singletons and for PAPP-A 2.192 for dichorionic and 1.788 for monochorionic twins were not implemented yet<sup>39</sup>.

### • • • Additional first trimester ultrasound markers

Additional first trimester ultrasound markers, such as nasal bone, abnormal ductus venosus flow, and tricuspid regurgitation allow even more specific fetal assessment for aneuploidy. In general, additional first trimester ultrasound markers can be used in two strategies, firstly, assessment in all patients or as second stage assessment in those patients with intermediate test results.

In singleton pregnancies the addition of nasal bone (NB) assessment has resulted in an increased DR (85-93%) at a 5% SPR for DS<sup>42,43</sup>. The screening performance of first trimester combined test with NB was also evaluated in 4188 twin fetuses demonstrating an 87% DR using NT+NB at a 5% SPR<sup>44</sup>. Cicero et al. also reported on nasal bone measurement in 112 twin pregnancies, however, the screening performance of NB in twin pregnancies was not addressed separately<sup>42</sup>. Abnormal flow in the ductus venosus has been reported in the first trimester in 3.7% of euploid singleton fetuses compared with 69.1% of DS fetuses<sup>45</sup>. Studies on ductus venosus flow in twin pregnancies are merely focussed on screening for Twin-to-Twin Transfusion syndrome (TTTS) and not as marker for DS. Maiz et al. studied 695 twin pregnancies and found a similarly high prevalence of abnormal ductus venosus flow in aneuploid compared with euploid twin fetuses as in singleton pregnancies (70% vs 7.7%,  $p < 0.001$ ). The screening performance of single NT measurements for aneuploidy was improved by adding ductus venosus flow<sup>46</sup>.

Besides the use for aneuploidy screening, abnormal ductus venosus flow in at least 1 monochorionic fetus at 11-13 weeks increases the risk on subsequent TTTS development.<sup>46-48</sup> Tricuspid regurgitation is observed in 0.9% of euploid singleton fetuses compared with 55.7% in DS fetuses. Screening performance for aneuploidy can be improved by adding tricuspid regurgitation to the current first trimester test.<sup>49</sup> Currently, no specific studies on tricuspid regurgitation and aneuploidy screening in twin pregnancies have been conducted.

### • • • Other chromosomal anomalies: trisomy 18 and 13

Sparse data are available on trisomy 13 and trisomy 18 screening in twin pregnancies. There is only one report addressing trisomy 18 screening in twin pregnancies with the current first trimester combined test. In this study single NT measurements detected 2 of the 3 trisomy 18 cases and first trimester combined test all three cases at 5% FPR.<sup>37</sup>

## ADVERSE PREGNANCY OUTCOME

• • • Hypertension/small for gestational age fetus/premature delivery  
Women pregnant with twins are at threefold increased risk of developing gestational hypertension and preeclampsia.<sup>50,51</sup> Premature delivery is also a major complication observed in twin pregnancies. Moreover, fetal growth restriction is more frequent in twin pregnancies than in singletons.<sup>52,53</sup> Current first trimester serum markers from the DS screening program have been reported in relation to adverse pregnancy outcome in singletons such as preeclampsia, fetal growth restriction, stillbirth and preterm birth.<sup>54,55</sup> For adverse pregnancy outcome in twins these relationships have been reported in only two studies. In 326 twin cases Chasen et al. reported on the association between low PAPP-A levels (5th percentile= $<0.52$  MoM) and subsequent development of discordant fetal growth and hypertension.<sup>56</sup> One study (n=70 twin pregnancies) reported on a relationship between low free  $\beta$ -hCG levels ( $\leq 25$ th percentile) and preterm delivery before 32 weeks of gestation.<sup>57</sup> Further studies are necessary to evaluate the current first trimester DS markers as predictors of adverse outcome in twin pregnancies.

### • • • Twin-to-Twin Transfusion Syndrome

In monochorionic twins the risk of adverse outcome is also determined by the existence of Twin-to-Twin Transfusion syndrome (TTTS), complicating approximately 9-15% of all monochorionic twin pregnancies.<sup>58-60</sup> TTTS occurs through vascular anastomoses in the shared monochorionic placenta, which are nearly always present but lead in TTTS to haemodynamic imbalance. Without intervention, TTTS often leads to either severe morbidity, mostly associated with preterm birth, or demise of one or both fetuses.<sup>61</sup> Timely diagnosis of TTTS is beneficial for treatment options and outcome of the fetuses.<sup>62-64</sup> The association between increased nuchal translucency and TTTS was reported already more than ten years ago.<sup>58,65</sup> Kagan et al. (2007) described a relationship between NT discordance of more than 20% between fetuses (and not necessarily increased NT) and adverse outcome, namely fetal death and TTTS.<sup>66</sup> In chapter 7 of this thesis we

described the use of NT discordance (the absolute difference NT fetus 1 and fetus 2 of the largest measurement) as predictor for TTTS and for earlier diagnosis in a selected group of MC twins referred for DS screening. We think that standardized calculation of NT discordance should be incorporated in MC twins so that predictive values can be calculated in an unselected population.

Further in this thesis (chapter 8) we evaluated, whether first trimester maternal markers used for DS screening can be used as predictor of TTTS. For second trimester markers an association between increased levels and subsequent TTTS development has been described, however, for first trimester markers this was not studied earlier. In sixty TTTS cases, the free  $\beta$ -hCG MoM corrected for twin chorionicity was significantly increased i.e. 1.39 versus 0.98 in the second trimester serum compared with MC twins with uncomplicated outcome. For alpha-fetoprotein (AFP) there was a non-significant increase of 1.15 vs. 0.99 MoM in TTTS compared with uncomplicated MC twins.<sup>67</sup> In first trimester serum we found increased levels of both free  $\beta$ -hCG and PAPP-A in MC twins complicated by TTTS, although not significant.<sup>68</sup>

• • • New screening markers for aneuploidy and adverse pregnancy outcome  
Currently many research activities are focussed on the development of new screening markers to improve the performance of current screening tests for aneuploidy and adverse outcome. ADAM12s is a potential marker. In chapter 4 we describe the results of ADAM12s used as marker for fetal trisomy 13, 18 and 21 in singleton pregnancies and found that ADAM12s was reduced especially in early first trimester for all trisomies. However, the screening performance of the combined test with addition of ADAM12s was not greatly improved.<sup>69</sup> In chapter 6 reference values of ADAM12 in euploid twin pregnancies and in DS cases are evaluated. We found increased ADAM12s MoM (1.61 MoM, n=209) in euploid twins compared with singletons however, not doubled like other first trimester markers such as free  $\beta$ -hCG and PAPP-A.<sup>70</sup> ADAM12s levels were significantly lower in monochorionic than in dichorionic twins, comparable to other first trimester markers free  $\beta$ -hCG and PAPP-A.<sup>39-41</sup> Since the publication of our study, one study was published shortly afterwards also addressing ADAM12s in twin pregnancies. They found similar median MoM values for ADAM12s, however, no differences in MoM values based on chorionicity.<sup>71</sup>

A statement about the screening performance of ADAM12s in twin trisomy cases is too early. In total 4 DS cases have been reported so far and ADAM12s values were found reduced in only two cases.<sup>70,71</sup>

Reduced ADAM12s levels have also been reported in singleton pregnancies with adverse pregnancy outcome: preeclampsia and/or fetal growth restriction.<sup>72-74</sup> In chapter 5 we reported on ADAM12s as marker for adverse pregnancy outcome, hypertensive disorders and small for gestational age fetuses (SGA), in singletons. We found reduced ADAM12s values for all outcomes, however, only significantly reduced in singletons complicated by gestational hypertension. In chapter 6 median ADAM12s MoM values in twin pregnancies with complicated outcome are evaluated. MoM values are not significantly different in those pregnancies complicated by hypertensive disorders or SGA fetus compared with uncomplicated twin pregnancies, making ADAM12s not a sufficient potential marker for predicting adverse pregnancy outcome in twins.<sup>70</sup>

Another potential marker for adverse pregnancy outcome is Placental Protein 13 (PP13). PP13 is a small dimer protein involved in implantation and spiral artery modification<sup>75</sup>. Previous studies have shown a relationship of low serum PP13 levels and subsequent development of preeclampsia in singleton pregnancies<sup>76,77</sup>. One report failed to show a significant relationship between low levels of PP13 and fetal growth restriction<sup>78</sup>. In chapter 5 we evaluated PP13 in singleton pregnancies with adverse outcome. Results were not so promising, since PP13 was decreased in all outcome groups, however, not significantly. Further studies are necessary in order to report on the performance of PP13 in predicting adverse outcome in twin pregnancies. Up till now only one study has reported on median MoM PP13 values in euploid twin pregnancies, namely 1.56 MoM in monochorionic (n=51) and 1.53 dichorionic (n=249). However, in this study data on outcome of the pregnancy are lacking<sup>71</sup>.

## CONCERNS AND CO-VARIABLES

Prenatal screening is complicated and in twin pregnancies even more complicated. In general it is important to note that in prenatal screening 'threshold' MoM values are used to discriminate between an affected and unaffected fetus. Serum markers are expressed in MoM values rather than in absolute concentrations, and follow a log normal distribution. The 'threshold' MoM values are different per individual marker.

In twin pregnancies fetuses can either be discordant (only one affected) or concordant (both affected) for both aneuploidy and structural anomalies. In twin pregnancies four theoretical situations are possible in prenatal screening: 1. both fetuses are unaffected 2. both fetuses are affected, 3. only fetus 1 is affected and finally 4. only fetus 2 is affected. Taking the foregoing into consideration the question raises how to report test results to the parents in twin cases: a fetus specific or pregnancy specific risk, or should the report be adjusted depending on chorionicity? In monochorionic twins studies have proposed to use the average NT to calculate a pregnancy specific risk<sup>8,21</sup>. Although monozygotic twins are considered genetically 'identical' a small number of discordances for chromosomal abnormalities has been described<sup>56</sup>. Moreover, several authors report on dissimilar NT values in monochorionic twin pairs concordant for a chromosomal abnormality<sup>21,58,79</sup>. Instead of the average NT in MC we advocate the use of the NT between-fetus correlation coefficient as a co-variable for both MC and DC twins. Individual NT measurements and fetus specific risk estimations allow identifying the specific fetus at risk and are thus considered in this thesis as most appropriate.

Overall availability of data on discordant and concordant DS twin cases raises some concern. The largest reports on DS twins, both concordant and discordant, in second trimester are 20 cases by Spencer and additionally 27 cases by Garchet al.<sup>16,34</sup>. For first trimester cases there is a review by Spencer dealing with 19 DS (both discordant and concordant) in which the reported free  $\beta$ -hCG twin-corrected MoM was 1.39 and PAPP-A 0.56 MoM<sup>34</sup>. The largest report on first trimester cases consist of 47 twin DS cases<sup>41</sup>. Effort should be made to combine all twin data on DS affected pregnancies internationally. Such initiative will lead to a better integration of all DS twin data to optimize risk estimation algorithms.

Screening programs are more and more improving towards individualised risk estimations taken into account co-variables and individual factors influencing maternal serum marker levels. Individual adjustment for gestational age, conception mode, and ethnicity are some co-variables to be listed. Twin screening is already complex, but how to implement all these co-variables? In singletons the use of the crown rump length (CRL) is advocated above clinical dating to correct serum markers. Spencer et al. (2008) uses the largest CRL to correct biochemical markers in twin pregnancies<sup>39</sup>. However, this method is neither evaluated nor motivated in any way. Probably, since serum and NT are measured at the same day in a one stop clinic model this method is opted for, contrary to the Dutch situation in which serum is usually taken earlier in pregnancy and often before NT measurement. Then the question rises whether it might be better to correct the serum values to the overall pregnancy date for example based on the average CRL from an early first trimester dating scan. Since free  $\beta$ -hCG levels normally decrease and PAPP-A levels increase in the first trimester of pregnancy, using the largest CRL (theoretically this can be more gestational days than clinical dating would predict) could account for unjust higher free  $\beta$ -hCG MoM and unjust lower PAPP-A MoM and thus providing a possible false positive test result. Moreover, it is interesting to discuss how to correct serum for pregnancies conceived by assisted reproduction. First trimester serum markers have been reported to be changed in ART conceived singleton pregnancies<sup>80</sup>. ART accounts for a substantial part of all twins, not only dizygotic twinning due to super ovulation and multiple embryo transfer but also monozygotic twinning<sup>81</sup>. So far in naturally conceived versus ART twins no significant differences in free  $\beta$ -hCG and PAPP-A are reported<sup>29,33</sup>. The latter finding was confirmed for IVF and ICSI twins in this thesis chapter 2<sup>40</sup>. A next possible co-variable is ethnicity. Ethnicity influences biochemical marker distributions, especially Afro-Caribbean and Asian women show higher PAPP-A and free  $\beta$ -hCG levels<sup>82</sup>. In this thesis the studied population is predominantly Caucasian. Studies on serum levels were therefore not corrected for ethnicity. In more diverse population correcting for ethnicity is advocated since the incidence of twinning is higher among Afro-Americans<sup>52</sup>.

## CONCLUSIONS

In conclusion, prenatal screening in twins is complex. Overall, it is advised to report on a fetus specific risk rather than on a pregnancy specific risk for both mono- and dichorionic twins. In general first trimester screening is advocated above second trimester screening and in any case above screening based on maternal age only. Single NT screening is a sufficient screening program in multiple pregnancies with comparable performance as in singletons. However, first trimester combined testing is reported to reduce false positive rates. For the future, screening programs need to incorporate the between-fetus correlation coefficient for NT measurements. Moreover, shifting from single NT measurements towards first trimester combined screening requires implementation of serum chorionicity correction factors. Additionally, other co-variables such as gestational age, conception mode and ethnicity need to be further evaluated in twin pregnancies to adapt the individual risk estimation. Likewise additional first trimester ultrasound markers will need to be evaluated for aneuploidy screening in twins, since these markers allow calculating an even more specific risk per fetus. Moreover, we think that calculation of NT discordance should be standardized in monochorionic twins so that predictive values can be calculated in an unselected population. Finally, the current serum screening markers of the first trimester combined test and new serum screening markers need to be evaluated as predictors of adverse pregnancy outcome in twin pregnancies.

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## Summary & Samenvatting

