CEOG Clinical and Experimental Obstetrics & Gynecology

Circulatory levels of tumor necrosis factor-α, interleukin-6, and syncytiotrophoblast microvesicles in the first trimester of pregnancy

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Summary

Background: The first trimester of pregnancy remains an unknown immunological adaptation. Cytokines and syncytiotrophoblast microvesicles (STBM) have been identified as key factors involved in the maternal immune adaptation. This study therefore aims to determine the levels of T helper cell 1 (Th1) and 2 (Th2) associated cytokines TNF-α and IL-6 in relation to the relative concentration of STBM's in maternal circulation. *Materials and Methods:* Plasma samples from normotensive pregnant women in the first trimester of pregnancy were obtained. The concentrations of TNF-α and IL-6 were determined using ELISA. STBMs were determined by isolating microvesicles in maternal circulation and quantifying the concentration of PLAP using ELISA. *Results:* TNF-α, IL-6 and STBMs remained at constant levels in weeks 5-10 of gestation. In weeks 11-12 of gestation, TNF-α increased with a decrease in IL-6 and STBMs. Ratio of TNF-α/IL-6 remained constant in weeks 5-6 and significantly increased in weeks 11-12 of gestation. A positive correlation between TNF-α and IL-6 was obtained in weeks 6-10 and a negative correlation in weeks 11-12 of gestation. In addition, a positive correlation between STBMs & TNF-α was obtained and negative correlation between STBMs and IL-6. was observed *Conclusion:* The relationship between TNF-α/IL-6 and STBMs suggests that syncytiotrophoblast microvesicles may have a role in cytokine production and in the maintenance of the Th1/Th2 immune adaptation in normal pregnancy.

Key words: IL-6; TNF-α; Syncytiotrophoblast microvesicles; Early pregnancy.

Introduction

Pregnancy is well recognised as a complex immunological adaptation between mother and fetus [1]. There are a multitude of factors such as placental-derived microvesicles, hormones, and cytokines [2] that synergistically coordinate the maternal immune adaptation in the first trimester of pregnancy, a prerequisite for successful pregnancy. The exact mechanism whereby these factors regulate this process is still unknown although more recent evidence suggests that placental-derived microvesicles may be implicated in the maternal immune adaptation, required for a successful pregnancy [3].

There are distinct fetal and maternal physiological developments that occur in the first trimester of pregnancy. These developments include placentation, embryogenesis, organogenesis, and pregnancy-associated cellular differentiation, which together form part of the co-ordinated natural phenomenon. In the first trimester of pregnancy, trophoblast cells invade the maternal decidua to ensure proper spiral artery remodelling, a tightly regulated process whereby any deviations from the normal could result in complicated or failed pregnancies [4]. These trophoblast cells are modulated by placental factors such as cytokines, hormones, and microvesicles. In particular, syncytiotrophoblast microvesicles (STBMS) are understood to play a role in maternal immune adaptation and systemic inflammatory response (MSIR) during normal and complicated pregnancies [5, 6].

Syncytiotrophoblast microvesicles consisting of subclasses of molecules ranging from 10-1000 nm in size include three main types of vesicles namely: (1) vesicles that bud directly from the cell membrane, (2) exosomes that are derived from multi-vesicular bodies within the cell, and (3) apoptotic bodies [5]. These microvesicles have been shown to have an immune regulatory role in pregnancy and are involved in the acceptance of the semi-allogenic fetus [7-9]. Importantly, STBM's have been shown to induce cytokine release in monocytes and B-cells [5] via cellular signalling cascades leading to the synthesis of inflammatory cytokines such as TNF-α and IL-6 [2]. In-vitro and ex-vivo studies

Revised manuscript accepted for publication August 31, 2017

Table 1. — *Clinical characteristics of patients.*

Variables	Gestational age (weeks)					
	5 (n = 3)	6 (n = 5)	8 (n = 12)	10 (n = 15)	11 (n = 15)	12 (n = 15)
Maternal age (years)	31.33 ± 4.81	26.83 ± 2.04	24.83 ± 2.19	25.16 ± 0.92	25.20 ± 1.27	25.91 ± 1.27
Parity	1 ± 0.33	1 ± 0.37	1 ± 0.29	1 ± 0.20	1 ± 0.15	1 ± 0.36
Systolic/diastolic Blood Pressure (mmHg)	121.3/83.33 ± 1.33/0.8	122.8/80.67 ± 1.64/2.19	123.2/81.92 ± 0.91/0.56	123.5/82.50 ± 0.60/0.34	124.7/82.65 ± 1.22/0.64	123.6/82.82 ± 1.47/0.63

All values are represented by mean ± SEM. All pregnancies were singleton without intrauterine infection or any other medical condition. Proteinuria was not detected in all patient groups. No statistical significance was identified with parity and blood pressure ($p > 0.05$).

have shown that both TNF-α and IL-6 have a synergistic role in regulating placental morphogenesis by co-ordinating trophoblast proliferation, migration, differentiation, and secretory function [10-12]. Additionally, ex-vivo studies have shown that STBMs stimulate the production of cytokines and therefore have an immune modulatory role in pregnancy. Syncytiotrophoblast microvesicles could therefore be responsible for the shift in Th1/Th2 immune adaptation in normal and complicated pregnancies [5]. It has been established that a shift from type 1 (Th1) to type 2 (Th2) immunity is an essential requirement for normal pregnancy and failure to make this shift results in a compromised or failed pregnancy [7]. The classical Th1/Th2 cytokine paradigm in pregnancy is a universally accepted concept even though placental-derived factors in relation to the maternal immune adaptation are not fully understood [13].

Previous studies have focused on maternal systemic levels of TNF-α and IL-6 in the second and third trimesters of pregnancy without determining the relative concentration of STBMs in maternal circulation [14-16]. In this study, the authors therefore aim to identify the possible relationship between maternal circulatory levels of cytokines (TNF-α and IL-6) and STBMs in the first trimester of pregnancy, the "black-box" period of pregnancy.

Materials and Methods

Regulatory ethical and institutional approval were obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, South Africa (BE036/12). All patients were recruited by informed consent.

Primiparous women in the first trimester of pregnancy at 5, 6, 8, 10, 11, and 12 weeks of gestation (n = 3, 5, 12, 15, 15, and 15, respectively). Normotensive pregnant women were classified by a blood pressure of 120±10 / 80±5 (systolic/diastolic mmHg) and absent proteinuria. Women had singleton pregnancies and those with evidence of any infections or medical, surgical or other obstetric complications were excluded. Blood samples were collected and the plasma samples were stored at -80°C for analyses.

Plasma concentrations of TNF-α and IL6 were measured using commercially available sandwich enzyme-linked immunosorbent assays (TNF-α & IL6 quantification kits). Briefly, specific monoclonal antibody (TNF-α/IL-6) was immobilised onto each well overnight at 4°C using the antibody binding buffer supplied. To prevent non-specific binding; wells were washed and 200 µl of assay diluent was added to each well and incubated at room temperature for one hour with agitation. Plates were thereafter washed with buffer and 100 µl of diluted plasma sample or standards were added to each well. Antigens were allowed to bind to primary antibodies at room temperature for two hours with agitation. Plates were washed with buffer followed by addition of 100 µl of HRP detection antibody into each well and incubated at room temperature for one hour with agitation. Plates were thereafter washed with buffer and 100 µl of Avidin-HRP was added to each well and incubated at room temperature for 30 minutes with agitation. Plates were washed and 100 µl of TMB substrate solution was added to each well and incubated for 15 minutes with no exposure to light. The reaction was monitored for colour change and stopped using the stop solution provided. The absorbance was read at 450 nm.

Microvesicles in maternal circulation was isolated according to a modified method as described by Dragovic *et al.* [17]. Microvesicle protein concentration was determined using a protein assay. Placental alkaline phosphatase concentration in maternal circulation was measured using a sandwich ELISA test. Plates were pre-coated with specific PLAP primary antibodies by the manufacturer. Approximately 25 µg of isolated microvesicles were added to each well and incubated at 37°C for 30 minutes. Plates were thereafter washed and 50 µl of HRP-conjugate was added to each well followed by incubation at 37°C for 20 minutes. Plates were thereafter washed and substrate solution A (50 µl) and B (50 µl) was added to each well and incubated at room temperature for ten minutes with no exposure to light. The reaction was terminated by the addition of 50 µl of stop solution and the absorbencies were read at 450 nm.

The data is presented as mean ± SEM. Differences in STBMs, IL6 and TNF-α concentration between pregnant women were determined using ANOVA with post-hoc analyses (Tukey-Kramer, 95% CI). Statistical analysis was performed using Prism 6. Correlation analysis was done using the Pearson's correlation test. Statistical significance was defined as $p < 0.05$.

Results

The clinical characteristics of the study groups are represented in Table 1. There was no statistical significance in the clinical data between the groups ($p > 0.05$).

The concentration of TNF-α and IL-6 in maternal circulation are represented in Figures 1A and B, respectively. TNF-α and IL-6 levels during early pregnancy (5-10 weeks) remained constant with no statistically significant differences ($p > 0.05$). A significant increase in TNF-α in weeks 11 (8.056 ± 0.15 pg/ml) and 12 (8.46 ± 0.19 pg/ml) was observed in comparison to weeks 5 (7.004 ± 0.20 pg/ml), 6 (7.345 ± 0.120), 8 (7.122 ± 0.12 pg/ml), and 10 (7.36 ± 0.05 pg/ml), $p < 0.05$. Conversely, the IL-6 levels significantly decrease in weeks 11 (14.32 ± 0.75 pg/ml) and 12 (13.47 ± 0.45 pg/ml) in comparison to week 10 (15.75

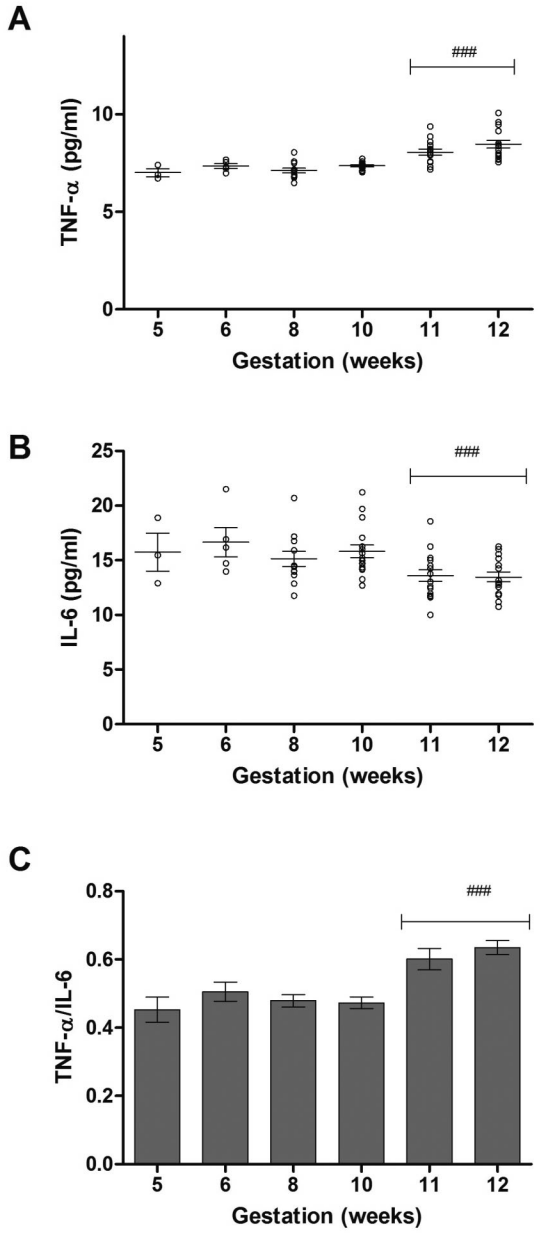

Figure 1. — Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in maternal circulation. Plasma (A) TNF-α (pg/ml) and (B) IL-6 (pg/ml) concentration measured by enzyme linked immunoabsorbency assay and (C) ratio of TNF-α to IL-6 in pregnant women at 5, 6, 8, 10, 11, and 12 weeks of gestation. In A and B data is presented as an aligned dot plot. In C data is presented as a bar graph. Values are represented as mean ± SEM. In A, B, and C $^{###}$ $p < 0.01$ 11 and 12 weeks *vs.* 5 to 10 weeks.

± 1.73 pg/ml) and 6 (16.65 ± 1.32 pg/ml), $p < 0.05$. The ratio of TNF-α to IL-6 in maternal circulation was calculated (Figure 1C). This ratio significantly increased in weeks 11 (0.60 ± 0.03) and 12 (0.64 ± 0.02) in comparison to weeks 5 (0.45 ± 0.04), 6 (0.49 ± 0.03), 8 (0.48 ± 0.02), and 10 (0.47 ± 0.02), $p < 0.05$.

The relationship between TNF-α and IL-6 in pregnant women was further analysed using Pearson's correlation coefficient. The analysis showed a positive correlation in weeks 6 ($r = 0.90$, $p < 0.05$; Figure 2A) and 10 ($r = 0.72$, $p < 0.01$; Figure 2B) of gestation. In contrast, a negative correlation was obtained in 11 ($r = -0.56$, $p < 0.05$; Figure 2C) and 12 ($r = -0.92$, $p < 0.05$; Figure 2D) weeks of gestation.

The differences in the relative concentration of STBMs in maternal circulation are shown in Figure 3. It is noted that the relative STBM concentration remained constant in weeks 5-10 (averages -905 ± 83.74 pg/ml) of pregnancy. However, a decrease in weeks 11 (508.9 ± 35.66 pg/ml) and 12 (556.7 ± 54.91 pg/ml) in comparison to weeks 5-10 was observed ($p < 0.05$).

Pearson's correlation coefficient analysis was used to further analyse the relationship between the relative STBM concentration and inflammatory cytokines (TNF-α and IL-6) during the first trimester of pregnancy. No correlation was obtained in the 8th week of gestation. However, the analysis indicates a positive correlation between relative STBM concentration and TNF-α in weeks 6 ($r = 0.96$, $p < 0.05$; Figure 4A), 10 ($r = 0.55$, $p < 0.05$; Figure 4B), 11 ($r = 0.59$, $p < 0.05$; Figure 4C) and 12 ($r = 76$, $p < 0.01$; Figure 4D) of gestation. In contrast, a negative correlation between relative STBM concentration and IL-6 in weeks 6 ($r = -0.92$, $p < 0.05$; Figure 4E), 10 ($r = -0.79$, $p < 0.01$; Figure 4F), 11 ($r = -0.53$, $p < 0.05$; Figure 4G), and 12 ($r = -0.55$, $p < 0.05$; Figure 4H) of gestation was obtained.

Discussion

In this study, the authors quantified cytokines TNF-α and IL-6 relative to the concentration of STBMs in maternal circulation over the first trimester of pregnancy, an underreported phase of pregnancy due to participant recruitment challenges. The study, therefore, provides data regarding the maternal circulatory levels of cytokines relative to STBMs in the first trimester of pregnancy. The main findings of the study showed constant levels of TNF-α, IL-6, and STBM levels in weeks 5-10 of pregnancy. However, in comparison, at weeks 11 and 12, TNF-α increases while IL-6 and STBM decline. Further analysis of the data showed an increased ratio of TNF-α/IL-6 (Th1/Th2) in weeks 11 and 12 compared to weeks 5-10. Correlation analysis between TNF-α and IL-6 displayed a positive correlation in weeks 6 and 10. Conversely, a negative correlation was observed in weeks 11 and 12, suggesting a slight shift towards Th1 immunity. In addition, the constant positive correlation between STBMs and TNF-α and negative correlation between STBM and IL-6 suggests that STBMs are associated with upregulating Th1 immunity and downregulating Th2 immunity in the first trimester of pregnancy. These findings are indicative of an alteration in immune response during the transitioning from organogenesis (5-10 weeks) to fetal development (≥11 weeks).

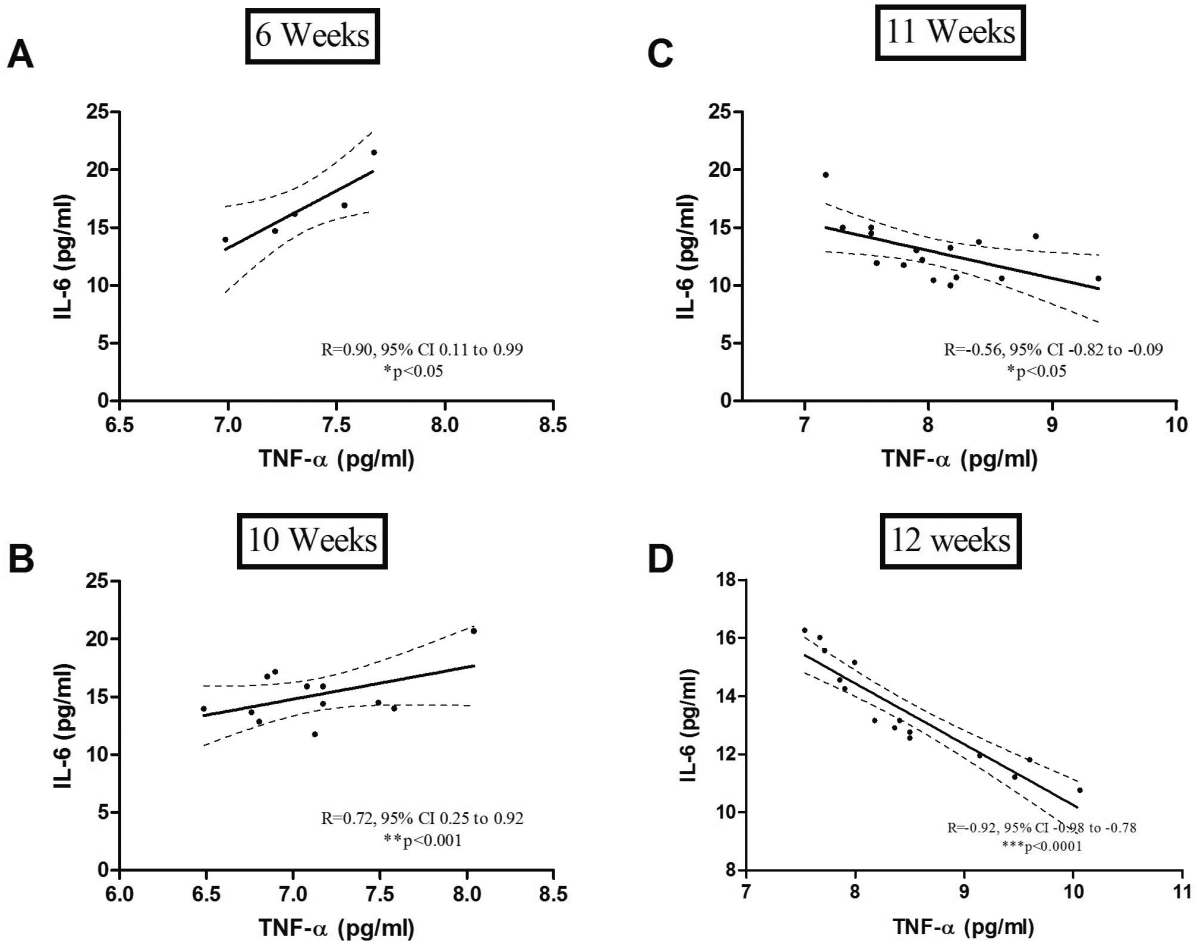

Figure 2. — The relationship between tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels in maternal circulation. The correlation between TNF-α and IL-6 in (A) 6, (B) 10, (C) 11, and (D) 12 weeks of gestation. In A and B a (+) Pearson's correlation. In D and E a (-) Pearson's correlation.

The maintained levels of TNF-α and IL-6 in weeks 5-10 of gestation is in keeping with a previous study which showed that there are no significant alterations in serum TNF-α and IL-6 levels during weeks 8-10 of gestation [18]. This could be representative of the maternal immune adaptation during organogenesis. Previous studies also showed that TNF-α and IL-6 increases in the second and third trimesters of normal pregnancy [14, 16, 18], which is a requirement for the maintenance of Th2 associated immunity. However, in this study, for the first time, the authors have shown that there is an increase in TNF-α and decrease in IL-6 in weeks 11 and 12 of gestation, which represents a slight shift towards Th1 immunity, which could be representative of the transition from organogenesis to fetal development. This finding was further assessed by the analysis of the TNF-α (Th1-pro-inflammatory)/IL-6 (Th2-anti-inflammatory) ratio. The ratio analysis supports the notion that in normal pregnancy there is a shift towards Th2 immunity, which is a common feature of successful pregnancy [19]. However, observations made in this study suggest that there is a greater shift towards Th2 immunity during weeks 5-10 of gestation in comparison to weeks 11 and 12. The slight shift towards Th1 immunity in weeks 11 and 12 of gestation probably occurs due to the oxygen demand for rapid fetal growth, which results in enhanced inflammation to support the increase in trophoblast invasion and vessel transformation; a critical requirement for spiral artery remodelling [4]. These findings support the theory that placental morphogenesis requires both a pro- and anti-inflammatory state during the first trimester of pregnancy [20]. It has been recognised that STBMs, released into maternal circulation could play a regulatory role in Th1/Th2 immunity which may mediate the synthesis of pro- and anti-inflammatory cytokines in maternal circulation [5].

Elevated STBMs in maternal circulation have been associated with pregnancy-related complications, such as preeclampsia and is indicative of placental function [21, 22]. Placental alkaline phosphatase, a syncytiotrophoblast membrane-bound allosteric enzyme, has been used to

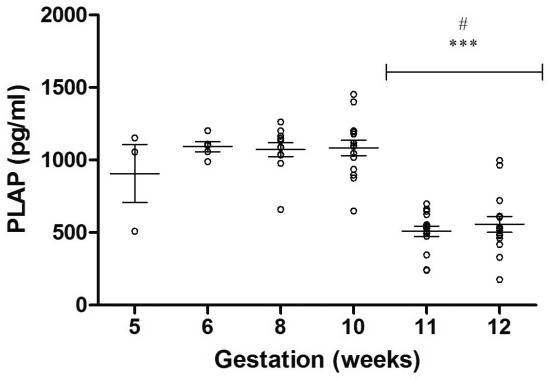

Figure 3. — Syncytiotrophoblast microvesicle concentration in maternal circulation. Syncytiotrophoblast microvesicles were quantified by the measurement of PLAP using enzyme linked immunoabsorbency assay in pregnant women at 5, 6, 8, 10, 11, and 12 weeks of gestation (n = 3, 5, 12, 15, 15, and 15, respectively). Data is presented as an aligned dot plot and values are represented as mean ± SEM. 11 and 12 weeks *vs.* 5 weeks ($^{\#}p < 0.05$), 6-10 weeks ($^{***}p < 0.01$).

measure the relative concentration of STBMs in maternal circulation, mainly in the 2nd and 3rd trimester of pregnancy. There is therefore a lack of information regarding the relative concentration of STBMs in 5-12 weeks of normal pregnancy. In this study, the authors therefore isolated and quantified circulating STBMs by the measurement of PLAP (stimulus) and related it to the concentration of TNF-α and IL-6 (maternal inflammatory response) in maternal circulation.

More recently ex-vivo studies showed that STBMs play a central role in promoting Th2 immunity during normal pregnancy and therefore could possibly play a central role maintenance of MSIR in normal and complicated pregnancies [5]. In this study, maternal circulatory levels of STBMs in weeks 5-10 of gestation remained constant with a significant decrease in weeks 11-12 of gestation ($p < 0.05$), which may occur as a result of the transitioning from organogenesis to fetal growth as discussed above.

The decrease in the ratio between TNF-α/IL-6 in weeks 5-10 is probably as a result of the increase in relative STBM concentration which may have stimulated the Th2 anti-inflammatory response leading to the increased synthesis of IL-6 required for facilitating fetal organ development [11]. In contrast, the rapid decline in STBMs in weeks 11-12 of gestation may have slightly shifted the inflammatory response towards Th1 immunity in comparison to a stronger Th2 response in weeks 5-10 of gestation. This is probably due to the synthesis of pro-inflammatory cytokines (TNF-α) from monocytes which were primed by STBMs earlier in pregnancy [5]. Further correlation analysis between STBMs (magnitude of the stimulus) related to TNF-α and IL-6 (size of the response) in maternal circulation during weeks 5-12 of gestation. The analysis demonstrates that STBMs are positively associated with TNF-α and negatively associated with IL-6 in the first trimester of pregnancy. These findings suggest that STBMs in the first trimester of pregnancy is positively associated with Th1 immunity. This is contrary to previous findings whereby STBMs derived in the third trimester have been shown to stimulate Th2 immunity [5] and could possibly be due to key immunological adaptations that occur throughout the various phases of gestation which ultimately affect the biogenesis of STBMs. In addition, it is important to note that the measurement of PLAP from isolated microvesicles may not be a true representation of STBMs in maternal circulation as STBMs consist of various types of vesicles. However, the measurement of PLAP from isolated microvesicles in maternal circulation would represent the relative concentration of STBMs in maternal circulation and may therefore be a useful tool in monitoring pregnancy when combined with pregnancy associated factors such as TNF-α and IL-6.

Since STBMs consist of sub-classes of vesicles, it is therefore necessary that future studies incorporate the isolation and characterisation of individual sub-classes of STBMs from maternal circulation. Consequently, studies regarding placental-derived exosomes [23] as biomarkers of obstetric complications are ongoing within the present research group. Nonetheless, the findings from this study indicate that combined analysis of TNF-α/IL-6 ratio and STBMs in maternal circulation can be incorporated into a medical algorithm to improve and standardise decisions made regarding normal and complicated pregnancies.

Conclusions

TNF-α (Th1), IL-6 (Th2) and STBM levels in maternal circulation remain constant in weeks 5-10 of gestation; a period of organogenesis. However, in weeks 11-12 of gestation, TNF-α increases with a decline in IL-6 and STBMs, which is suggestive of the physiological transitioning from organogenesis to fetal development as a result of enhanced placental perfusion. Ratio analysis of TNF-α/IL-6 indicates that there is an enhanced shift towards Th1 immunity in weeks 11 and 12 of gestation; this may occur in support of placental spiral artery remodelling, a perquisite for fetal growth. Additionally, the measurement of the relative concentration of STBMs in relation to TNF-α and IL-6 indicates that STBMs may play a key role in the immune adaptation in early pregnancy. This study shows that combined analysis of TNF-α, IL-6, and STBMs may provide a useful medical algorithm in ascertaining successful pregnancy and impeding obstetric complications in the future.

Acknowledgments

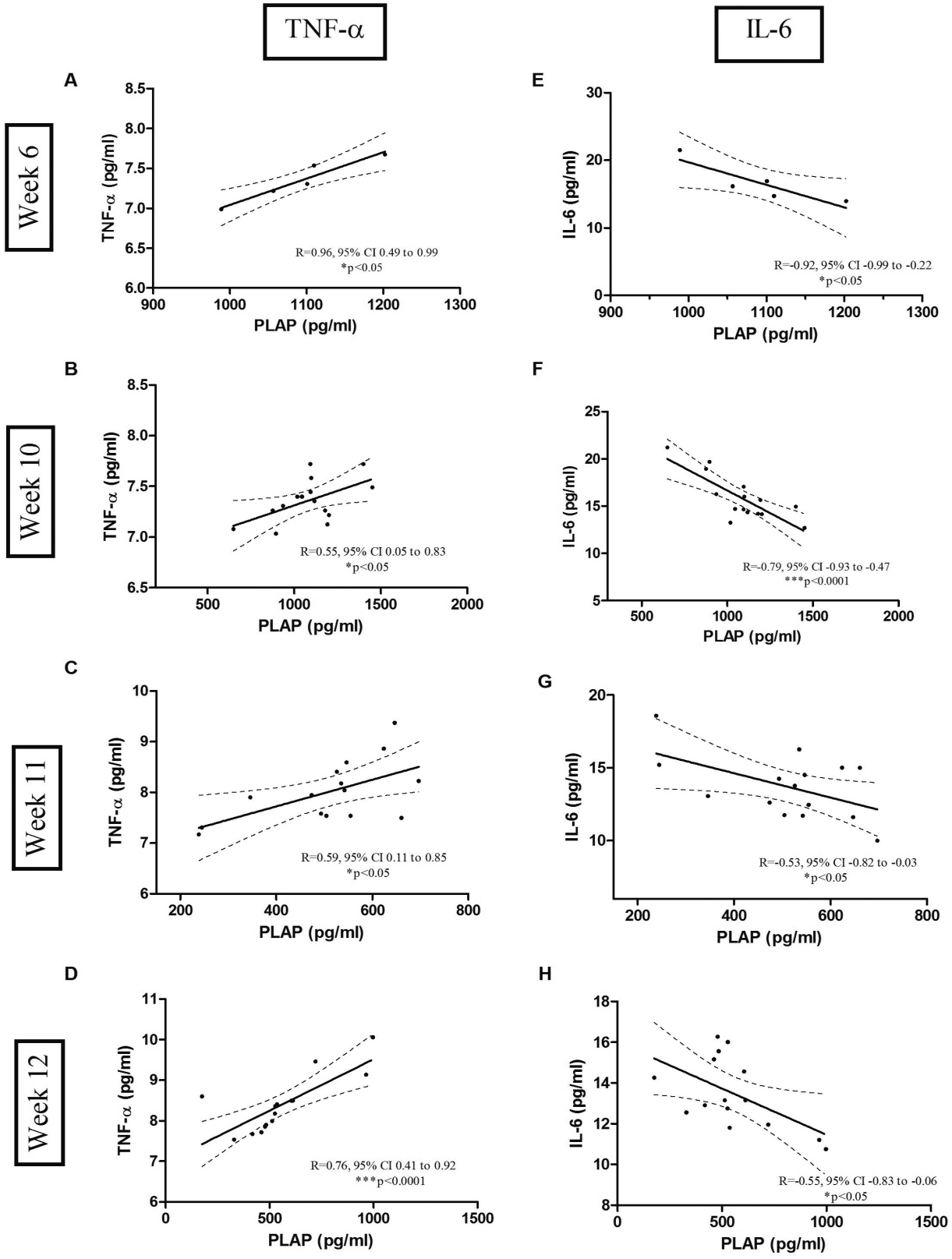

Figure 4. — The relationship between tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and syncytiotrophoblast microvesicles in maternal circulation. The correlation between relative concentration of STBMs by the measurement of PLAP (pg/ml) and TNF-α in weeks (A) 6, (B) 10, (C) 11, and (D) 12 of gestation; IL-6 in weeks (E) 6 and (F) 8, (G) 11, and (H) 12 of gestation. In A, B, C, and D a (+) Pearson's correlation. In E, F, G, and H a (-) Pearson's correlation.

The authors thank Dr. Niren Maharaj and Dr. Prem Gathiram for their assistance and Miss Z. Mkhize for the collection of blood samples. This project was funded by the National Research Foundation of South Africa (Grant Number: 91544).

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