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Changes in cytokine and sequestosome-1 levels during twin pregnancy progression: Association with outcome

Angela Silvano [a], Giovanni Sisti [b], Viola Seravalli [a], Noemi Strambi [a], Astrid Parenti [c], Amedeo Amedei [d], Steven S. Witkin [e,f], Mariarosaria Di Tommaso [a,*]

[a] Department of Health Sciences, Division of Obstetrics and Gynecology, Careggi Hospital, University of Florence, Florence, Italy
[b] Division of Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, College of Medicine, University of Arizona, USA
[c] Department of Health Sciences, Clinical Pharmacology and Oncology Section, University of Florence, Italy
[d] Department of Clinical and Experimental Medicine, University of Florence, Italy
[e] Department of Obstetrics and Gynecology, Weill Cornell Medicine, NY, USA
[f] Department of Infectious Diseases and Parasitology, Laboratory of Virology, University of Sao Paulo Faculty of Medicine, Sao Paulo, Brazil

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ABSTRACT

Background: Twin pregnancies are associated with complications and adverse outcomes. The number of twin pregnancies has increased in the last decades, due to the use of assisted reproductive techniques and delayed childbearing. Analysis of changes that occur during twin pregnancy progression and their association with outcome will lead to improved clinical interventions.
Objective: We evaluated if the plasma concentration of select cytokines and the level of sequestosome-1 (p62) in peripheral blood mononuclear cells (PBMCs) during each trimester of twin gestations was predictive of pregnancy outcome.
Study design: This prospective, observational study was conducted at Careggi University Hospital, Florence, Italy. Plasma from 82 women with twin pregnancies was collected in each trimester for measurement of interleukin (IL)-1β, IL-6, IL-10, IL-12 and tumor necrosis factor (TNF)-α. The intracellular PBMC concentration of p62, a protein involved in autophagy, kinase activity and cell differentiation, was also determined.
Results: IL-1β ($p < 0.001$), IL-6 ($p < 0.001$), TNF-α ($p < 0.001$) and p62 ($p < 0.05$) increased from the 1st to the 2nd to the 3rd trimester. The TNF-α level was correlated with the IL-1β concentration in the 1st and 3rd trimesters $p < 0.01$) and with the IL-6 concentration in each of the three trimesters ($p < 0.01$). The intracellular p62 level in PBMCs was negatively correlated with the concentration of IL-1β in the 2nd trimester ($p < 0.05$) and negatively correlated with the IL-6 level in the 3rd trimester ($p < 0.05$). The TNF-α level was significantly higher in the 2nd ($p < 0.05$) and 3rd ($p < 0.001$) trimester in women with a spontaneous preterm delivery. The TNF-α concentrations in the 2nd ($p < 0.05$) and 3rd ($p < 0.01$) trimester, respectively, and 3rd trimester IL-6 ($p < 0.01$), were negatively associated with gestational age at delivery. The concentration of IL-6 was highest in the 2nd ($p < 0.05$) and 3rd ($p < 0.05$) trimesters in women who utilized assisted reproductive technologies. An elevated IL-1β level in the 3rd trimester was associated with gestational diabetes mellitus ($p < 0.05$).
Conclusion: Variations in cytokine levels between individual women during the three trimesters of twin gestations are predictive of spontaneous preterm delivery and the onset of gestational diabetes.

1. Introduction

Twin pregnancies are associated with multiple potential complications such as miscarriage, stillbirth, twin–twin transfusion syndrome (TTTS), congenital anomalies, abnormal placentation, fetal growth restriction and preterm birth (PTB).[1–8] The number of twin pregnancies has increased in recent decades, primarily due to the use of assisted reproductive technologies (ART) and delayed childbearing.

In response to infection or to a non-infectious disruption of cell homeostasis, cytokines such as interleukin (IL)-1β, IL-12 and tumor necrosis factor (TNF)-α are produced by lymphocytes and macrophages to initiate inflammation and negate the threat. IL-6, a pleiotropic cytokine

* Corresponding author.
E-mail address: mariarosaria.ditommaso@unifi.it (M. Di Tommaso).

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capable of pro- and anti-inflammatory activity and a sensitive biomarker of chronic inflammation[9] also increases. The anti-inflammatory cytokine IL-10 is subsequently produced to down-regulate the extent of pro-inflammatory immune activation. Effective regulation of the extent of pro-inflammatory immunity during gestation is essential for a healthy outcome. [10–13] Cytokine levels in maternal plasma[14,15] as well as in amniotic fluid[16] and cervicovaginal secretions[17,18] have been reported to be biomarkers of PTB in singleton gestations. However, there is no data on changes in cytokine concentrations through the three trimesters of twin pregnancies and their possible association with adverse outcomes.

Another regulator of cell homeostasis essential for successful pregnancy outcome is autophagy. [19,20] Aggregated or misfolded proteins, aged or damaged organelles as well as microbial components in the cytoplasm that disrupt normal cell functions become bound to a cytoplasmic protein, sequestosome-1 (p62) [21,22] and the complex is sequestered in a double-membraned structure called an autophagosome. [23,24] The autophagosome then combines with a lysosome and the engulfed components along with p62 are degraded by lysosomal enzymes. Thus, fluctuations in the cytoplasmic p62 concentration have been used as a biomarker for the extent of autophagy in specific cell types. [25–27] p62 has additional functions, such as the modulation of intracellular kinases and cell activation and differentiation[28–30] that are also essential for cytokine production and pregnancy progression. Disruptions of autophagy during gestation have been associated with complications such as preeclampsia[31,32], fetal growth restriction[33] and preterm delivery. [34,35] The inhibition of autophagy and activation in peripheral blood mononuclear cells (PBMCs) leads to a reduction in their ability to produce pro-inflammatory cytokines in response to infection or adverse non-infectious conditions. [36].

The aim of the present study was to measure changes in the levels of select pro- and anti-inflammatory cytokines in the circulation, as well as the level of p62 in PBMCs, in each trimester of twin gestations. Specifically, we evaluated their associations with the onset of gestational diabetes mellitus (GDM), spontaneous preterm birth and gestational age at delivery in spontaneous or ART-assisted conceptions in diamniotic (MCDA) and dichorionic diamniotic (DCDA) twin pregnancies.

2. Materials and methods

2.1. Ethical approval

The study was approved by the Ethical Committee of Azienda Ospedaliero-Universitaria Careggi (Ref. no.10255/2017).

2.2. Study design

This was a prospective, observational study conducted between January 2018 and February 2022 at Careggi University Hospital in Florence, Italy and involved women carrying a twin pregnancy who were followed at the Institution's Multiple Gestation Clinic. Both spontaneous and ART- conceived pregnancies, as well as dichorionic and monochorionic pregnancies, were included. Exclusion criteria included previous preterm birth, monochorionic- monoamniotic twin pregnancy, first or second trimester pregnancy loss, termination of pregnancy, major fetal anomalies, the presence of a cervical cerclage, and loss of follow-up from antenatal care. In our study cohort, there were no cases of chronic comorbidities or pregestational diabetes, and no TTTS were observed. In addition, there were no cases of maternal infection as determined by the absence of clinical symptoms in mothers or newborns.

Blood samples were collected at the first, second and third trimester of pregnancy. The range of gestational ages at sampling was from 8 weeks to 15 weeks in the 1st trimester and from 17 to 26 weeks, and from 26 weeks and 2 days to 35 weeks in the 2nd and 3rd trimester of pregnancy, respectively.

In pregnancies conceived spontaneously, the gestational age was calculated from the last menstrual period and confirmed by the fetal crown-rump length (CRL) measurement from the first trimester ultrasound. The larger of the two CRLs was used to estimate gestational age. Twin pregnancies conceived via ART were dated using the embryonic age from the time of fertilization. Reasons for ART included occluded Fallopian tubes, anovulation or inadequate sperm parameters in the male partners. Chorionicity was determined before 13 weeks and 6 days of gestation using the membrane thickness at the site of insertion of the amniotic membrane into the placenta. [37] All women provided written informed consent before inclusion.

2.3. Diagnosis of gestational diabetes

Gestational diabetes mellitus (GDM) was diagnosed based on a fasting plasma glucose $\geq$ 5.1 mmol/L, and/or 1-h plasma glucose $\geq$ 10.0 mmol/L, and/or 2-h plasma glucose $\geq$ 8.6 mmol/L in the 75 g oral glucose tolerance test. [38].

2.4. Plasma cytokine determination

Blood samples (6–8 ml) were collected in each trimester by venipuncture into tubes containing EDTA and centrifuged at 2000 RPM for 10 min. Plasma was stored in one ml aliquots at −80° C before use in the cytokine assays. IL-1β, IL-6, IL-10, IL-12 and TNF-α levels were evaluated using a Milliplex custom kit, Human Cytokines panel A for Luminex MAGPIX detection system (Affymetrix, eBioscience), following the manufacturers' instructions.

2.5. PBMC isolation and processing

PBMCs were isolated using Ficoll-Paque density-gradient centrifugation (Lympholyte®-H; Cedarlane Laboratories, Hornby ON, Canada). They were collected, washed with sterile phosphate-buffered saline pH 7.2 with 1 % fetal bovine serum, and 2×10^6 cells were lysed with ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 1 % Triton X-100, 10 mM β-glycerophosphate,1 mM sodium fluoride, 1 mM sodium orthovanadate, EDTA, 1 mM of a DNase (DNase, Roche, Basel, Switzerland), protease inhibitor cocktail and 0.2 mM phenylmethylsulfonyl fluoride. p62 was quantified in lysed PBMCs samples by a commercial Enzyme-Linked Immunosorbent Assay, following the manufacturer's instructions (p62 ELISA KIT; ENZO Life Sciences New York, USA). The lower limit of detection was 100 pg/ml.

2.6. Statistical analysis

The Mann-Whitney test, the Spearman rank correlation test and Friedman pairwise comparison were performed, as appropriate, to evaluate differences in cytokine and p62 levels over time, the relation between individual cytokine concentrations or the p62 level and their association with clinical outcome. A p value < 0.05 was considered significant. The statistical analysis was performed using the IBM SPSS Statistics, version 28.

3. Results

One hundred and fifteen women with twin pregnancies consented to participate in the study. Of these, 33 met one or more of the exclusion criteria. Therefore, 82 women were included in the analysis.

Patients' demographics, clinical information and pregnancy outcomes are described in Table 1. The majority of the women (95 %) were White, 2.5 % were Black and 2.5 % were Asian. The median [interquartile range (IQR)] age of the women was 35 (33, 38) years and their median (IQR) body mass index was 21.5 (19.6, 23.9) kg/m^2. Pregnancy was spontaneous in 63.4 % of the women while 36.6 % conceived with the aid of various ARTs. Dichorionic twin pregnancies were present in

Table 1
Patient demographic and clinical information. BMI, body mass index; IUI, intrauterine insemination; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm insemination; IQR, interquartile range. Spontaneous preterm birth is defined as delivery before 37 weeks gestation.

Clinical characteristics	Pregnant women
Number of enrolled pregnant women	82
Ethnicity	
White	78 (95.1 %)
Black	2 (2.4 %)
Asian	2 (2.4 %)
Gravidity	
0	45 (54.9 %)
1	30 (36.6 %)
2	4 (4.8 %)
3	2 (2.4 %)
4	1 (1.2 %)
Parity	
0	57 (69.5 %)
1	21 (25.6 %)
2	3 (3.6 %)
3	1 (1.2 %)
Age at sampling (years) median (IQR)	35 (33,38)
BMI kg/m^2 median (IQR)	21.5 (19.6,23.9)
Assisted reproductive technology	30 (36.6 %)
IUI	2 (6.6 %)
IVF	5 (16.6 %)
ICSI	21 (70.0 %)
Egg donation	2 (6.6 %)
Gestational age at sampling in 1st trimester (weeks) mean ± SD	12.20 ± 2.0
Gestational age at sampling in 2nd trimester (weeks) mean ± SD	22.0 ± 0.2.2
Gestational age at sampling in 3rd trimester (weeks) mean ± SD	31.0 ± 2.0
Gestational diabetes mellitus	17 (21.0 %)
Monochorionic-diamniotic twin pregnancy	19 (23.2 %)
Dichorionic-diamniotic twin pregnancy	63 (76.8 %)
Gestational age at birth (weeks) mean ± SD	36.0 ± 1.6
Spontaneous preterm birth	9 (11 %)
1st Baby sex	
Male	41 (50 %)
Female	41 (50 %)
2nd Baby sex	
Male	50 (61 %)
Female	32 (39 %)
Median	
1st Baby weight (gr) (IQR)	2310 (2005,2620)
2nd Baby weight (gr) (IQR)	2400 (2060,2630)

76.8 % of the women while 23.2 % had monochorionic-diamniotic twin gestations. The mean (SD) gestational age at delivery was 36.3 (2.0) weeks and 11.0 % had a spontaneous preterm birth, defined as delivery before 37 completed weeks of gestation. The timing of delivery, term or preterm, was due primarily to individual variations on the influence of two neonates on uterine and cervical stress parameters. There was no evidence of infection-related preterm birth. Similarly, there was no relation between reason for ART and pregnancy outcome.

The sex of the first delivered baby was 50 % male and 50 % female, while the second baby was 61 % male and 39 % female. The median (IQR) weight of the first baby was 2310 (2005, 2620) g while the second baby was 2400 (2060, 2630) g. GDM was detected in 21.0 % of the mothers. The mean (SD) gestational age at blood collection was 12.2 (2.0) weeks in the first trimester, 22.0 (2.2) weeks in the second trimester and 31.0 (2.0) weeks in the third trimester.

3.1. Plasma cytokines and intracellular p62 concentrations in twin pregnancies

Variations in circulating cytokine levels and intracellular p62 concentrations in PBMCs during the course of the twin pregnancies are shown in Table 2. Median concentrations of circulating IL-1β, IL-6 and TNF-α increased with each subsequent semester ($p < 0.001$), while levels of IL-10 and IL-12 remained unchanged. The median concentration of p62 in PBMCs also progressively increased as pregnancy progressed ($p = 0.028$).

Correlations between levels of individual cytokines and between cytokines and p62 in each trimester are shown in Table 3. The levels of TNF-α were correlated with the levels of IL-1β in the 1st and 3rd trimesters ($p < 0.01$) and with the IL-6 level in each of the three trimesters ($p < 0.01$). The intracellular p62 level in PBMCs was negatively correlated with the concentration of IL-1β in the 2nd trimester ($p < 0.05$) and negatively correlated with the IL-6 level in the 3rd trimester ($p < 0.05$).

Women with different ethnicities, dichorionic or monochorionic pregnancies or reason for undergoing ART did not vary in the levels of cytokines detected in each trimester.

Table 4 evaluates the association between plasma cytokines and p62 in PBMCs in each trimester and spontaneous preterm birth. The concentration of TNF-α was significantly higher in the 2nd ($p = 0.043$) and 3rd ($p < 0.001$) trimester in women who subsequently spontaneously delivered preterm, as compared to those with a term delivery. None of the other cytokines or p62 was associated with spontaneous preterm birth in any trimester. The 36 women with iatrogenic preterm births were excluded from this analysis. In addition, there was a negative association between the TNF-α concentration in the 2nd (rho = −0.27, p = 0.015) and 3rd (rho = −0.34, $p < 0.002$) trimester, respectively, and gestational age at delivery. An elevated IL-6 level in the 3rd trimester was similarly correlated with a lower gestational age at delivery (rho = −0.34, $p < 0.002$).

The relationship between cytokine and p62 concentrations in each trimester and whether conception was spontaneous or utilized ART is shown in Table 5. Only the concentrations of IL-6 differed by means of conception and were higher in the 1st ($p = 0.021$) and 2nd ($p = 0.029$) trimesters in women who utilized ART.

In terms of development of GDM, an elevation only in the IL-1β level in the 3rd trimester was associated with GDM ($p = 0.022$). The median (IQR) IL-1β concentration was 7.1 (5.1, 11.4) pg/ml in women who did not develop GDM vs. 10.9 (7.9, 12.5) pg/ml in those diagnosed with GDM during their pregnancy.

4. Discussion

4.1. Principal findings

As gestation advances, maternal tissues experience increasing fetal demand and are subject to enhanced physical stress. This is especially apparent when one than a single neonate is present. The present study

Table 2
Concentration of circulating cytokines and intracellular p62 in PBMCs during each trimester of twin pregnancies. Values are pg/ml for cytokines and ng/ml for p62. Differences in concentration between the three trimesters was analyzed by the Friedman test. NS, not significant.

Compound	Median concentration (IQR)			
	1st trimester	2nd trimester	3rd trimester	p value
IL-1β	3.2 (1.7, 5.6)	5.5 (3.7, 8.6)	7.6 (5.5, 12.1)	<0.001
IL-6	0.3 (0.2, 0.4)	0.3 (0.2, 0.6)	0.6 (0.3, 1.10	<0.001
IL-10	2.1 (1.3, 3.8)	2.2 (1.3, 3.8)	2.3 (1.1, 4.0)	NS
IL-12p70	0.9, (0.9, 1.0)	0.9 (0.9, 1.0)	0.9 (0.9, 1.3)	NS
TNF-α	7.1 (5.0, 11.3)	7.5 (5.4, 11.4)	10.1 (7.1, 15.6)	<0.001
p62	9.0 (6.2, 13.5)	10.2 (7.2, 15.5)	15.5 (9.7, 20.7)	0.028

Table 3
Associations between different cytokines and between cytokines and p62 during different trimesters in twin pregnancies. NS, not significant.

Pair	Spearman rho, p value		
	1st trimester	2nd trimester	3rd trimester
IL-1β-TNF-α	0.31, 0.005	0.26, <0.05	0.30, < 0.01
IL-β-IL-6	0.31, <0.05	0.37, <0.001	0.27, <0.05
TNF-α-IL-6	0.45, <0.01	0.35, 0.001	0.51, <0.01
IL-1β-p62	NS	−0.28, <0.05	NS
TNF-α-p62	NS	NS	NS
IL-6-p62	NS	NS	−0.25, <0.05

Table 4
Association between plasma cytokines and p62 in PBMCs in each trimester and spontaneous preterm birth. Thirty-six women with iatrogenic preterm birth were excluded from the analysis. SPTB, spontaneous preterm birth; TB, term birth.

Compound	Trimester	Median concentration (IQR)		
		SPTB	TB	
		N = 11	N = 35	p value
IL-1β	1st	3.1 (1.2, 7.9)	3.2 (1.7, 5.4)	NS
	2nd	5.0 (3.1, 8.6)	5.9 (4.0, 7.9)	NS
	3rd	10.1 (4.8, 12.5)	7.5 (4.8, 10.9)	NS
TNF-α	1st	9.2 (6.0, 11.3)	7.0 (3.9, 12.1)	NS
	2nd	11.3 (7.0, 14.5)	6.9 (3.9,8.5)	0.043
	3rd	16.8 (10.0, 20.1)	8.5 (6.2, 12.1)	<0.001
IL-6	1st	0.3 (0.2, 0.4)	0.3 (0.2, 0.4)	NS
	2nd	0.3 (0.2, 0.4)	0.3 (0.2, 0.6)	NS
	3rd	0.7 (0.3, 1.2)	0.5 (0.3, 0.8)	NS
p62	1st	11.3 (7.3, 21.6)	8.3 (5.7, 11.3)	NS
	2nd	10.6 (9.9, 14.0)	9.4 (6.7, 14.6)	NS
	3rd	15.6 (12.8, 21.9)	17.4 (10.6, 22.7)	NS

Table 5
Association between plasma cytokines and p62 in PBMCs in each trimester and spontaneous or ART conception.

Compound	Trimester	Median concentration (IQR)		
		Spontaneous	ART	
		N = 54	N = 28	p value
IL-1β	1st	3.2 (1.4, 5.7)	3.1 (1.9, 5.7)	NS
	2nd	5.4 (3.7, 9.5)	5.9 (3.6, 8.5)	NS
	3rd	7.1 (5.2, 11.7)	9.2 (6.1, 12.9)	NS
TNF-α	1st	7.3 (650, 11.5)	6.6 (4.5, 11.1)	NS
	2nd	8.0 (5.4, 11.7)	7.3 (5.1,8.5)	NS
	3rd	10.8 (7.6, 15.9)	8.7 (6.7, 12.7)	NS
IL-6	1st	0.3 (0.2, 0.3)	0.4 (0.2, 0.5)	0.021
	2nd	0.3 (0.2, 0.5)	0.5 (0.3, 0.9)	0.029
	3rd	0.5 (0.3, 1.0)	0.7 (0.5, 1.2)	NS
p62	1st	8.2 (5.8, 13.2)	10.0 (7.1, 14.2)	NS
	2nd	10.2 (6.6, 15.1)	11.0 (7.9, 25.0)	NS
	3rd	16.9 (9.7, 21.7)	12.6 (6.8, 19.1)	NS

demonstrates that concentrations of IL-1β, IL-6 and TNF-α sequentially increase in parallel during each trimester of a twin pregnancy. This indicates that pro-inflammatory immune activity is elevated as gestation progresses. Furthermore, the greatest increases were associated with pregnancy complications. The highest levels of IL-6 and TNF-α were associated with a lower gestational age at delivery and with a spontaneous preterm birth. Elevated IL-1β levels were associated with development of GDM. It thus appears that a lack of efficient regulation of the extent of pro-inflammatory immune activation, and/or individual variations in the extent of an elevated stress response to twin neonates predisposes towards adverse outcomes in twin gestations.

There was also a parallel increase with gestational age in the intracellular p62 concentration in PBMCs, indicating a decrease in autophagy and/or an elevation in kinase activity and activation of mononuclear immune cells as pregnancy progresses. The negative association between the intracellular p62 level and the circulating concentrations of IL-1β and IL-6 levels are consistent with the possibility that deficits in intracellular regulatory mechanisms result in elevated pro-inflammatory cytokine production and the increased likelihood of an adverse pregnancy outcome.

4.2. Results in the context of what is known

IL-1, IL-6, and TNF-α, produced by multiple immune system cells, including macrophages, dendritic cells, granulocytes, T and B lymphocytes during pregnancy, optimize embryo growth and development by modulating pro-inflammatory immune responses. Additionally, trophoblast cells release IL-1 and TNF-α, and endothelial cells release IL-6, within the uterus to further regulate local immunity within the uterus. [13] Consequently, these cytokines act both locally and systemically to mediate events that can adversely influence pregnancy outcome. [13,14,16].

Our observations are consistent with previous findings. Elevated circulating concentrations of IL-6, TNF-α, and IL-1β have been associated with spontaneous preterm birth in singleton pregnancies. [39–44] Similarly, elevated IL-1β production has been reported to inhibit insulin signalling in human trophoblasts. [45] This provides an explanation for our observation of an association between the IL-1β level and GDM in our subjects. IL-6 levels in the circulation and in follicular fluid have been shown to be elevated in response to ovarian hyperstimulation. [46,47] These findings are consistent with our observation of elevated IL-6 in the 1st and 2nd trimester in women undergoing ART as compared to women with a spontaneous conception.

4.3. Research implications

Our results suggest that the measurement of changes in plasma cytokine levels and p62 in PBMCs in each trimester of pregnancy may increase our understanding of mechanisms of adverse events in pregnancy and, in addition, have predictive value of pregnancy-associated sequela. Future studies may identify additional biomarkers associated with specific adverse events, such as preeclampsia, intrauterine growth restriction, or spontaneous preterm birth. [48] Furthermore, understanding how cytokines interact with each other and with p62 will provide an elevated level of detection of mechanisms by which these interactions further influence pregnancy outcome.

4.4. Strengths and limitations

The use of a standardized protocol for patient selection, the collection of samples and for sample processing in a single dedicated laboratory limited the risk of technical artifacts. Such a study design contributed to reducing the number of confounding variables.

Our study has limitations. Different ethnic groups are lacking in our study cohort. We have no information on vaginal microbiome composition. A recent study has demonstrated that prior pregnancy history influences the composition of the vaginal microbiota in women with singleton pregnancies. [49] Vaginal microbiome composition plays a major role in immune status and susceptibility to spontaneous preterm birth. [50,51] To our knowledge, the preterm births in our study population were due to individual variations in physical constraints on the uterus and cervix due to the presence of two neonates. Although there was no evidence of infection-related preterm delivery, as determined by the absence of maternal or neonatal fever, this ideally should be confirmed by more sophisticated analyses. Similarly, the absence of an apparent association between reason for undergoing ART and pregnancy outcome must be viewed as tentative due to our small patient population. Addressing these variables in future investigations should enhance the reliability of associations between cytokine and p62 levels and twin

pregnancy outcome parameters.

4.5. Clinical implications

This study demonstrated an association between alterations in cytokines and a PBMC component as twin pregnancies progressed and the occurrence of spontaneous preterm birth and GDM. These observations contribute to a broader understanding of the potential influence of quantitative variations in biomarkers that are present during twin pregnancy progression and prediction of maternal and fetal outcomes.

4.6. Conclusions

In twin pregnancies, levels of circulating pro-inflammatory cytokines, and the p62 concentration in PBMCs, increase through the three trimesters. The greatest elevations in IL-1β, IL-6 and TNF-α levels, associated with a decrease in intracellular p62 concentrations, are associated with earlier parturition. Obtaining a better understanding of the mechanisms involved is desirable to more accurately predict the subsequent occurrence of adverse sequela and provide more timely and effective interventions to improve fetal and maternal outcomes.

CRediT authorship contribution statement

Angela Silvano: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Giovanni Sisti:** Writing – review & editing, Visualization, Methodology, Formal analysis, Conceptualization. **Viola Seravalli:** Writing – original draft, Visualization, Supervision, Project administration, Methodology. **Noemi Strambi:** Investigation, Data curation. **Astrid Parenti:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Conceptualization. **Amedeo Amedei:** Writing – review & editing. **Steven S. Witkin:** Writing – review & editing, Visualization, Validation, Supervision. **Mariarosaria Di Tommaso:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mariarosaria Di Tommaso reports financial support was provided by Ente Cassa di Risparmio di Firenze. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

[1] B. Blondel, M. Kaminski, Trends in the occurrence, determinants, and consequences of multiple births, Semin. Perinatol. 26 (4) (2002) 239–249, https://doi.org/10.1053/sper.2002.34775.
[2] F.M. Russo, E. Pozzi, F. Pelizzoni, L. Todyrenchuk, D.P. Bernasconi, S. Cozzolino, P. Vergani, Stillbirths in singletons, dichorionic and monochorionic twins: a comparison of risks and causes, Eur. J. Obstet. Gynecol. Reprod. Biol. 170 (1) (2013) 131–136, https://doi.org/10.1016/j.ejogrb.2013.06.014.
[3] C. Hubinont, L. Lewi, P. Bernard, E. Marbaix, F. Debiève, E. Jauniaux, Anomalies of the placenta and umbilical cord in twin gestations, Am. J. Obstet. Gynecol. 213 (4 Suppl) (2015) S91–S102, https://doi.org/10.1016/j.ajog.2015.06.054.
[4] M.E. Paepe, Examination of the twin placenta, Semin. Perinatol 39 (1) (2015) 27–35, https://doi.org/10.1053/j.semperi.2014.10.005.
[5] M. Di Tommaso, V. Seravalli, S. Arduino, C. Bossotti, G. Sisti, T. Todros, Arabin cervical pessary to prevent preterm birth in twin pregnancies with short cervix, J. Obstet. Gynaecol 36 (6) (2016) 715–718, https://doi.org/10.3109/01443615.2016.1148127.
[6] F. D'Antonio, A.O. Odibo, F. Prefumo, et al., Weight discordance and perinatal mortality in twin pregnancy: systematic review and meta-analysis, Ultrasound. Obstet. Gynecol 52 (1) (2018) 11–23, https://doi.org/10.1002/uog.18966.
[7] M. Saito, M. Tokunaka, H. Takita, et al., Impact of first trimester determination of abnormal cord insertion on twin-to-twin transfusion syndrome and other adverse outcomes in monochorionic diamniotic twins: A retrospective cohort study, Prenat. Diagn 40 (4) (2020) 507–513, https://doi.org/10.1002/pd.5633.
[8] Y. Chitrit, D. Korb, C. Morin, T. Schmitz, J.F. Oury, O. Sibony, Perinatal mortality and morbidity, timing and route of delivery in monoamniotic twin pregnancies: a retrospective cohort study, Arch. Gynecol. Obstet 303 (3) (2021) 685–693, https://doi.org/10.1007/s00404-020-05782-1.
[9] M. Murakami, D. Kamimura, T. Hirano, Pleiotropy and specificity: Insights from the interleukin 6 family of cytokines, Immunity 50 (2019) 812–831, https://doi.org/10.1016/j.immuni.2019.03.027.
[10] J.R. Challis, C.J. Lockwood, L. Myatt, J.E. Norman, J.F. Strauss, F. Petraglia, Inflammation and Pregnancy, Reprod. Sci 16 (2) (2009) 206–215, https://doi.org/10.1177/1933719108329095.
[11] P. Hsu, R. Nanan, Foetal immune programming: hormones, cytokines, microbes and regulatory T cells, J. Reprod. Immunol 104–105 (2014) 2–7, https://doi.org/10.1016/j.jri.2014.02.005.
[12] S. Saito, A. Nakashima, T. Shima, M. Ito, Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy, Am. J. Reprod. Immunol 63 (6) (2010) 601–610, https://doi.org/10.1111/j.1600-0897.2010.00852.x.
[13] L.J. Yockey, A. Iwasaki, Interferons and proinflammatory cytokines in pregnancy and fetal development, Immunity 49 (3) (2018) 397–412, https://doi.org/10.1016/j.immuni.2018.07.017.
[14] A.E. Curry, I. Vogel, K. Skogstrand, et al., Maternal plasma cytokines in early- and mid-gestation of normal human pregnancy and their association with maternal factors, J. Reprod. Immunol 77 (2) (2008) 152–160, https://doi.org/10.1016/j.jri.2007.06.051.
[15] M. Kwiatek, T. Gęca, A. Kwaśniewska, Pro- and anti-Inflammatory cytokines in the first trimester-comparison of missed miscarriage and normal pregnancy, Int. J. Environ. Res. Public. Health 18 (16) (2021) 8538, https://doi.org/10.3390/ijerph18168538.
[16] S.M. Lee, J.S. Park, E.R. Norwitz, et al., Mid-trimester amniotic fluid pro-inflammatory biomarkers predict the risk of spontaneous preterm delivery in twins: a retrospective cohort study, J. Perinatol 35 (8) (2015) 542–546, https://doi.org/10.1038/jp.2015.29.
[17] Y. Hattori, T. Nakanishi, Y. Ozaki, K. Nozawa, T. Sato, M. Sugiura-Ogasawara, Uterine cervical inflammatory cytokines, interleukin-6 and -8, as predictors of miscarriage in recurrent cases, Am. J. Reprod. Immunol 58 (4) (2007) 350–357, https://doi.org/10.1111/j.1600-0897.2007.00516.x.
[18] J. Skrzypczak, P.K. Wirstlein, M. Wróbel, M. Mikołajczyk, Concentration of pro-inflammatory interleukins in cervical secretions of women with PROM and in the umbilical cord blood of their newborns, Ginekol. Pol. 86 (6) (2015) 434–441, https://doi.org/10.17772/gp/2400. Polish.
[19] D. Glick, S. Barth, K.F. Macleod, Autophagy: cellular and molecular mechanisms, J. Pathol 221 (1) (2010) 3–12, https://doi.org/10.1002/path.2697.
[20] X. Zhao, Y. Jiang, T. Jiang, et al., Physiological and pathological regulation of autophagy in pregnancy, Arch. Gynecol. Obstet 302 (2) (2020) 293–303, https://doi.org/10.1007/s00404-020-05607-1.
[21] Y. Katsuragi, Y. Ichimura, M. Komatsu, p62/SQSTM1 functions as a signaling hub and an autophagy adaptor, FEBS. J 282 (24) (2015) 4672–4678, https://doi.org/10.1111/febs.13540.
[22] G. Bjørkøy, T. Lamark, S. Pankiv, A. Øvervatn, A. Brech, T. Johansen, Monitoring autophagic degradation of p62/SQSTM1, Methods. Enzymol 452 (2009) 181–197, https://doi.org/10.1016/S0076-6879(08)03612-4.
[23] Y. Zhang, S.K. Calderwood, Autophagy, protein aggregation and hyperthermia: A mini-review, Int. J. Hyperthermia 27 (5) (2011) 409–414, https://doi.org/10.3109/02656736.2011.552087.
[24] T. Orfanelli, J.M. Jeong, G. Doulaveris, K. Holcomb, S.S. Witkin, Involvement of autophagy in cervical, endometrial and ovarian cancer, Int. J. Cancer 135 (3) (2014) 519–528, https://doi.org/10.1002/ijc.28524.
[25] Y. Ichimura, M. Komatsu, Pathophysiological role of autophagy: Lesson from autophagy-deficient mouse models, Exp. Anim 60 (4) (2011) 329–345, https://doi.org/10.1538/expanim.60.329.
[26] B. Levine, N. Mizushima, H.W. Virgin, Autophagy in immunity and inflammation, Nature 469 (7330) (2011) 323–335, https://doi.org/10.1038/nature09782.
[27] X. Xu, J. Ren, Unmasking the janus faces of autophagy in obesity-associated insulin resistance and cardiac dysfunction, Clin. Exp. Pharmacol. Physiol 39 (2) (2012) 200–208, https://doi.org/10.1111/j.1440-1681.2011.05638.x.
[28] F. Christian, E. Krause, M.D. Houslay, G.S. Baillie, PKA phosphorylation of p62/SQSTM1 regulates PB1 domain interaction partner binding, Biochim. Biophys. Acta 1843 (11) (2014) 2765–2774, https://doi.org/10.1016/j.bbamcr.2014.07.021.
[29] J. Moscat, M.T. Diaz-Meco, M.W. Wooten, Signal integration and diversification through the p62 scaffold protein, Trends. Biochem. Sci 32 (2) (2007) 95–100, https://doi.org/10.1016/j.tibs.2006.12.002.
[30] M.L. Seibenhener, T. Geetha, M.W. Wooten, Sequestosome1/p62–more than just a scaffold, FEBS. Lett 581 (2) (2007) 175–179, https://doi.org/10.1016/j.febslet.2006.12.027.

[31] S. Sharma, Autophagy-based diagnosis of pregnancy hypertension and pre-eclampsia, Am. J. Pathol 188 (11) (2018) 2457–2460, https://doi.org/10.1016/j.ajpath.2018.09.001.
[32] A. Nakashima, S.B. Cheng, M. Ikawa, et al., Evidence for lysosomal biogenesis proteome defect and impaired autophagy in preeclampsia, Autophagy 16 (10) (2020) 1771–1785, https://doi.org/10.1080/15548627.2019.1707494.
[33] T. Kojima, T. Yamada, R. Akaishi, et al., Role of the Atg9a gene in intrauterine growth and survival of fetal mice, Reprod. Biol 15 (3) (2015) 131–138, https://doi.org/10.1016/j.repbio.2015.05.001.
[34] B. Cao, C. Macones, I.U. Mysorekar, ATG16L1 governs placental infection risk and preterm birth in mice and women, JCI Insight 1 (21) (2016) e86654.
[35] K.M. Akram, L.I. Frost, D.O. Anumba, Impaired autophagy with augmented apoptosis in a Th1/Th2-imbalanced placental micromilieu is associated with spontaneous preterm birth, Front. Mol. Biosci 9 (2022) 897228, https://doi.org/10.3389/fmolb.2022.897228.
[36] T.T. Kanninen, A. Jayaram, S. Jaffe Lifshitz, S.S. Witkin, Altered autophagy induction by sera from pregnant women with pre-eclampsia: a case-control study, BJOG 121 (8) (2014) 958–964, https://doi.org/10.1111/1471-0528.12755.
[37] A. Khalil, M. Rodgers, A. Baschat, A. Bhide, E. Gratacos, K. Hecher, M.D. Kilby, et al., ISUOG Practice Guidelines: role of ultrasound in twin pregnancy, Ultrasound. Obstet. Gynecol. 47 (2) (2016) 247–263, https://doi.org/10.1002/uog.15821.cite.
[38] World Health Organization, Diagnostic criteria and classification of hyperglycaemia first detected in pregnancy, World Health Organization, 2013 https://apps.who.int/iris/handle/10665/85975.
[39] F. Lucaroni, L. Morciano, G. Rizzo, et al., Biomarkers for predicting spontaneous preterm birth: an umbrella systematic review, J. Matern. Fetal. Neonatal. Med 31 (6) (2018) 726–734, https://doi.org/10.1080/14767058.2017.1297404.
[40] L. Huang, Q. Hou, Y. Huang, et al., Serum multiple cytokines for the prediction of spontaneous preterm birth in asymptomatic women: A nested case-control study, Cytokine. 117 (2019) 91–97, https://doi.org/10.1016/j.cyto.2019.02.007.
[41] E. Amabebe, D.R. Chapman, V.L. Stern, G. Stafford, D.O.C. Anumba, Mid-gestational changes in cervicovaginal fluid cytokine levels in asymptomatic pregnant women are predictive markers of inflammation-associated spontaneous preterm birth, J. Reprod. Immunol 126 (2018) 1–10, https://doi.org/10.1016/j.jri.2018.01.001.
[42] M. Nadeau-Vallée, D. Obari, C. Quiniou, et al., A critical role of interleukin-1 in preterm labor, Cytokine. Growth. Factor. Rev 28 (2016) 37–51, https://doi.org/10.1016/j.cytogfr.2015.11.001.
[43] E.Y. Jung, J.W. Park, A. Ryu, S.Y. Lee, S.H. Cho, K.H. Park, Prediction of impending preterm delivery based on sonographic cervical length and different cytokine levels in cervicovaginal fluid in preterm labor, J. Obstet. Gynaecol. Res 42 (2) (2016) 158–165, https://doi.org/10.1111/jog.12882.
[44] O. Equils, C. Kellogg, J. McGregor, M. Gravett, G. Neal-Perry, C. Gabay, The role of the IL-1 system in pregnancy and the use of IL-1 system markers to identify women at risk for pregnancy complications, Biol. Reprod 103 (4) (2020) 684–694, https://doi.org/10.1093/biolre/ioaa102.
[45] I.L. Aye, T. Jansson, T.L. Powell, Interleukin-1β inhibits insulin signaling and prevents insulin-stimulated system A amino acid transport in primary human trophoblasts, Mol. Cell. Endocrinol 381 (1–2) (2013) 46–55, https://doi.org/10.1016/j.mce.2013.07.013.
[46] J.R. Loret de Mola, J.P. Flores, G.P. Baumgardner, J.M. Goldfarb, V. Gindlesperger, M.A. Friedlander, Elevated interleukin-6 levels in the ovarian hyperstimulation syndrome: ovarian immunohistochemical localization of interleukin-6 signal, Obstet. Gynecol. 87 (4) (1996) 581–587, https://doi.org/10.1016/0029-7844(95)00456-4.
[47] A. Younis, K. Hawkins, H. Mahini, W. Butler, M. Garelnabi, Serum tumor necrosis factor-α, interleukin-6, monocyte chemotactic protein-1 and paraoxonase-1 profiles in women with endometriosis, PCOS, or unexplained infertility, J. Assist. Reprod. Genet 31 (11) (2014) 1445–1451, https://doi.org/10.1007/s10815-014-0237-9.
[48] K.K. Hornaday, E.M. Wood, D.M. Slater, Is there a maternal blood biomarker that can predict spontaneous preterm birth prior to labour onset? A systematic review, PLoS. One. 17 (4) (2022 Apr 4) e0265853.
[49] R. Romero, K.R. Theis, N. Gomez-Lopez, A.D. Winters, J.J. Panzer, H. Lin, J. Galaz, et al., The vaginal microbiota of pregnant women varies with gestational age, maternal age, and parity, Microbiol. Spectr. 11 (4) (2023) e0342922.
[50] I. Kosti, S. Lyalina, K.S. Pollard, A.J. Butte, M. Sirota, Meta-analysis of vaginal microbiome data provides new insights into preterm birth, Front. Microbiol. 11 (2020) 476, https://doi.org/10.3389/fmicb.2020.00476.
[51] P. Pruski, G.D.S. Correia, H.V. Lewis, et al., Direct on-swab metabolic profiling of vaginal microbiome host interactions during pregnancy and preterm birth, Nat. Commun 12 (1) (2021) 5967, https://doi.org/10.1038/s41467-021-26215-w.