UNIVERSITY OF MEDICINE AND PHARMACY "CAROL DAVILA", BUCHAREST

DOCTORAL SCHOOL

MEDICINE FIELD

Ph.D. THESIS SUMMARY

Coordinator:

Assoc. Prof. Alexandru Filipescu, M.D., Ph.D.

Ph.D Student:

Nicoleta Mureanu, M.D.

Bucharest

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THE ROLE OF T CELLS SUBSETS IN PREGNANCY ADVERSE OUTCOME

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Introduction

Gestational diabetes mellitus (GDM) is a condition characterized by glucose intolerance that develops during pregnancy. It poses significant risks for both the mother and the fetus, leading to various adverse outcomes. Understanding these risks is crucial for effective management and prevention.

The motivation underlying the choice of the doctoral research topic consists in the fact that Gestational diabetes mellitus (GDM) affects approximately 13% of pregnancies worldwide, making it a significant public health concern (Zhang et al., 2016) and is associated with short- and long- term complications for the mother and child.

Gestational diabetes mellitus (GDM) is broadly defined as hyperglycaemia first recognised during pregnancy (McIntyre, H. D. *et al.* Gestational diabetes mellitus. *Nat Rev Dis Primers* **5**, 47 (2019).

GDM is one of the most common complications in pregnancy, affecting 9–26% of pregnancies worldwide, with a rapidly increasing global incidence (Jiang, L. *et al.* A global view of hypertensive disorders and diabetes mellitus during pregnancy. *Nat. Rev. Endocrinol.* **18**, 760–775 (2022), Sweeting, A., Wong, J., Murphy, H. R. & Ross, G. P. A Clinical Update on Gestational Diabetes Mellitus. *Endocr. Rev.* **43**, 763–793 (2022). Diagnosis of GDM is linked to clinical risk factors such as obesity, age, ancestry, and a family history of type 2 diabetes. However, no diagnostic threshold has been adopted globally (McIntyre, H. D. *et al.* Gestational diabetes mellitus. *Nat Rev Dis Primers* **5**, 47 (2019). Furthermore, hyperglycaemia may remain undiagnosed in patients who do not meet specific thresholds (American Diabetes Association. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2020. *Diabetes Care* **43**, S14–S31 (2020). GDM is associated with an increased risk of postpartum Type 2 Diabetes (Bellamy, L., Casas, J.-P., Hingorani, A. D. & Williams, D. Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. *Lancet* **373**, 1773–1779 (2009), Noctor, E. & Dunne, F. P. Type 2 diabetes after gestational diabetes: The influence of changing diagnostic criteria. *World J. Diabetes* **6**, 234–244 (2015), an increased risk of cardiovascular disorders (Sweeting, A., Wong, J., Murphy, H. R. & Ross, G. P. A Clinical Update on Gestational Diabetes Mellitus. *Endocr. Rev.* **43**, 763–793 (2022) and a greater risk of metabolic syndrome in offspring (Ornoy, A., Becker, M., Weinstein-Fudim, L. & Ergaz, Z. Diabetes during Pregnancy: A Maternal Disease Complicating the Course of Pregnancy with Long-Term Deleterious Effects on the Offspring.

A Clinical Review. *Int. J. Mol. Sci.* **22**, (2021). As such, GDM represents an ongoing global health challenge.

Early detection, effective management, and lifestyle modifications are crucial in reducing these risks and promoting healthier pregnancies. Ongoing research into the mechanisms and long-term effects of GDM will further enhance our understanding and ability to mitigate its impact.

The doctoral thesis is structured into two parts. The first part includes the latest data on epidemiology, risk factors, subtypes, screening, diagnosis and management as well as the impact of gestational diabetes in pregnancy and its potential adverse outcome. The special part includes the methodology, the analysis and results of the studies I have undergone with focusing on isolation and freezing of human peripheral blood mononuclear cells in pregnant patients, optimization of Peripheral Blood Mononuclear Cell (PBMC) isolation and flow cytometry. Over 2000 pregnant participants were recruited at 36 weeks of gestation and gave informed consent for their participation in the study and blood donation and analysed.

I. GENERAL PART

1. An overview - Definition, prevalence and risk factors

Diabetes Mellitus is a group of metabolic conditions involving elevated glucose levels related to issues in secretion and/or resistance of insulin, a hormone resealed by pancreatic cells whose role is to regulate the cellular uptake of blood glucose ("2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes—2021" 2021). The main categories of DM include autoimmune type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) which usually occurs on a background of insulin resistance, other types (diabetes due to pancreatitis or chemical-induced diabetes), and gestational diabetes mellitus (GDM) ("2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes—2021" 2021).

High blood sugar levels (hyperglicaemia) during pregnancy, whether resulting from pre-existing type 1 or type 2 diabetes or gestational diabetes, can lead to adverse outcomes for both the mother and the child(Egan et al., 2015). Historically, before the discovery of insulin in the early 20th century, women with diabetes were often advised against pregnancy due to the significantly high risks of maternal and neonatal mortality (approximately 50% for both) (Gabbe, 1992).

Gestational diabetes mellitus (GDM) affects approximately 13% of pregnancies worldwide, making it a significant public health concern (Zhang et al., 2016) and is associated with short- and long- term complications for the mother and child.

Gestational diabetes mellitus (GDM) is characterized by the body's inability to meet the heightened insulin demands typical of a normal pregnancy, leading to increased insulin resistance, glucose intolerance, and low-grade systemic inflammation (Kampmann, 2015).

Gestational diabetes mellitus (GDM) can develop during pregnancy due to various risk factors. Understanding these factors is crucial for early identification and management. Here are the primary risk factors associated with gestational diabetes:

- Obesity: Being overweight or obese (having a body mass index [BMI] of 30 or higher) before pregnancy significantly increases the risk of developing GDM.
- Age: Women over the age of 25 are at a higher risk, with the risk increasing further for those over 35.
- Family History: A family history of diabetes, particularly type 2 diabetes, can increase the likelihood of developing GDM.
- Previous Gestational Diabetes: Women who have had gestational diabetes in a previous pregnancy are at a higher risk of developing it again in subsequent

pregnancies or a family history of diabetes, and belonging to an ethnic group with a high risk of diabetes.

- Ethnicity: Certain ethnic groups, including African American, Hispanic/Latino, Native American, Asian American, and Pacific Islander populations, have a higher prevalence of GDM.
- Polycystic Ovary Syndrome (PCOS): Women with PCOS, a condition characterized by hormonal imbalances and insulin resistance, are at an increased risk of developing GDM.
- Sedentary Lifestyle: Lack of physical activity and a sedentary lifestyle can contribute to obesity and insulin resistance, increasing the risk of GDM.
- Unhealthy Diet: Diets high in refined carbohydrates, sugars, and unhealthy fats can lead to weight gain and insulin resistance, raising the risk of GDM.
- Multiple Pregnancies: Women carrying multiples (twins, triplets, etc.) are at a higher risk due to increased demands on the body and potential for greater weight gain.
- High Blood Pressure: A history of hypertension or high blood pressure can increase the risk of developing gestational diabetes.
- History of Macrosomia: If a previous baby was born weighing more than 9 pounds (greater than 4.5 kg),), the risk of GDM in subsequent pregnancies is higher.
- Certain Medical Conditions: Conditions such as gestational hypertension or preeclampsia in previous pregnancies can also increase the risk of GDM.

High-risk individuals are formally diagnosed with GDM based on criteria such as a fasting plasma glucose level of ≥5.6 mmol/L or a two-hour plasma glucose level of ≥7.8 mmol/L in a 75-g-2-hour oral glucose tolerance test conducted between 24-28 weeks of gestation (NICE 2015).

2. Adverse outcome of gestational diabetes for mother and child

Hyperglycaemia in pregnancy, whether from pre-existing type 1 or type 2 diabetes or due to gestational diabetes, poses the risk of adverse outcomes for both the mother and the child (Egan et al., 2015). In fact, prior to the discovery of insulin in the early 20th century, women with diabetes were generally warned against becoming pregnant due to the incredibly high risk of maternal and neonatal death (approximately 50% for both) (Gabbe, 1992). There are significant short-and long-term implications for both the mother and child as reported by

the large Hyperglycaemia and adverse pregnancy outcome (HAPO) study and several others ("Hyperglycemia and Adverse Pregnancy Outcomes," 2008). During pregnancy, women with GDM have an increased risk of developing gestational hypertension (28%) and preeclampsia (3.3%) (Kvetny & Poulsen, 2003); (Östlund et al., 2004) After pregnancy, there is a considerable risk of women developing type 2 diabetes (9.5%) (Vounzoulaki et al., 2020), (Shostrom et al., 2017). There is also other risk such as higher likelihood of caesarian delivery. GDM can lead to larger fetal size (macrosomia), which may necessitate a caesarian section to safely deliver the baby. After birth, infants of mothers with GDM may experience low blood sugar levels, which can lead to seizures and other health issues if not promptly addressed. They may have a higher risk of respiratory issues due to immature lung development, particularly if born preterm. It is well established that infants of mothers with GDM have an increased risk of developing metabolic disturbances, including both T1DM and T2DM, and mothers have an increased risk of developing GDM I subsequent pregnancies (Murray & Reynolds, 2020). Therefore, it is essential that screening for GDM and management interventions are implemented as early as possible to optimise maternal, foetal and child health and intercept the transgenerational cycle of non- communicable diseases.

3. Pathophysiology of GDM: role of immune cells

The pathophysiology of gestational diabetes mellitus (GDM) is complex and not fully understood, with evidence suggesting that it may develop over a substantial period of time. Research by (Lekva et al., 2016) indicates that the development of GDM may not be acute but rather dependent on long-term maternal factors. In many cases, the failure of pancreatic beta-cells to adequately compensate for a chronic excess of fuel can eventually lead to insulin resistance and hyperglycemia, as highlighted by (Plows et al., 2018). This failure of beta-cell function to keep up with the demands placed on them by increased insulin resistance is a key factor in the pathogenesis of GDM. Further research is needed to fully elucidate the mechanisms involved in the development and progression of GDM, as well as to identify effective strategies for prevention and management.

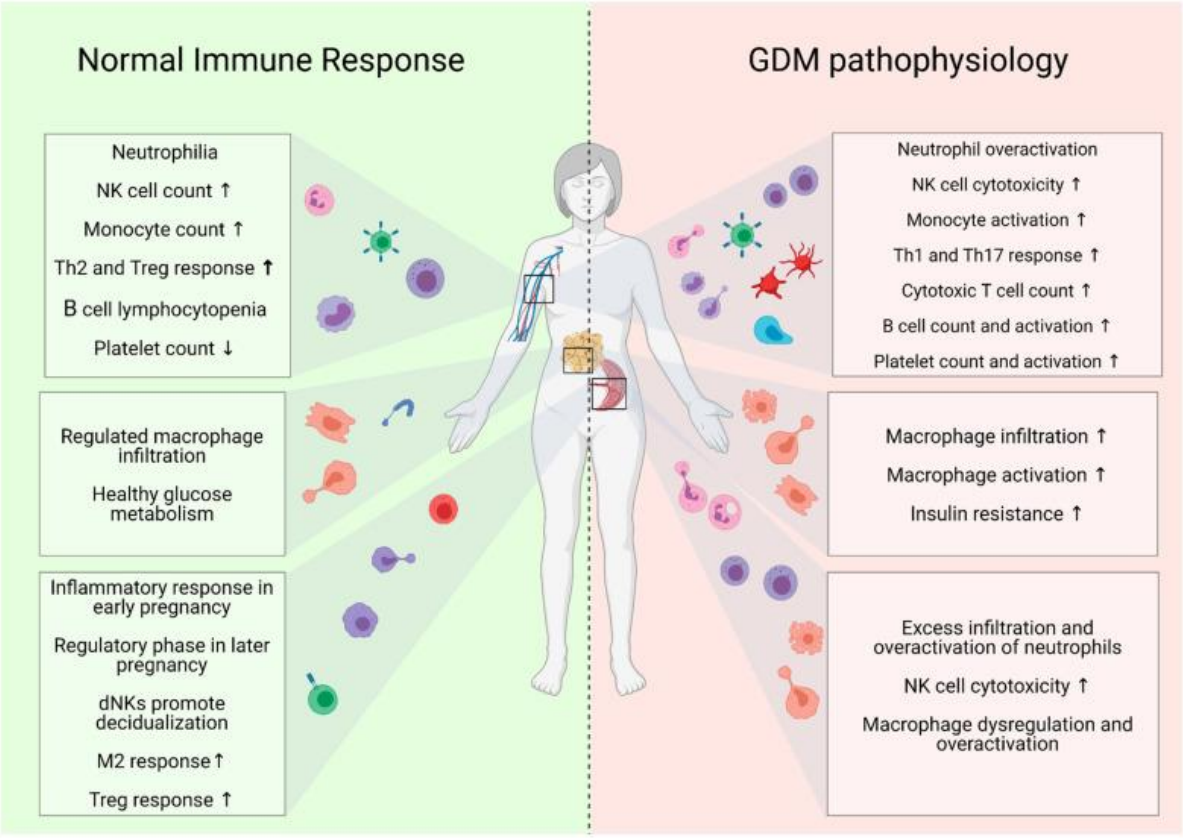

Figure 1. Summary and comparison between the changes of immune cells that occur in GDM with the changes that occur in the normal immune response. As seen, although there is an increased Treg response in the normal immune response, the response of Tregs in GDM is unknown. Taken and adapted from (McElwain et al., 2021a)

4. Regulatory T-cells (Tregs) and Gestational Diabetes

In a normal pregnancy, the immune system undergoes heightened adaptation to accommodate the developing fetus (McElwain et al., 2021b). However, in the case of gestational diabetes mellitus (GDM), characterized by low-grade systemic inflammation, the immune response may struggle to meet the increased demands, deviating from the typical immune profile observed in healthy pregnancies. Recent studies have focused on alterations in T-cell distributions in peripheral blood, potentially linked to the progression of GDM. This research aims to delineate the immune profile of T-cell subsets associated with GDM, shedding light on the immune landscape of this condition (SIFNAIOS et al., 2019). In healthy pregnancies, Tregs prevent the rejection of the fetus by the maternal immune system Krop, J., Heidt, S., Claas, F. H. J. & Eikmans, M. Regulatory T Cells in Pregnancy: It Is Not All About FoxP3. *Front. Immunol.* **11**, 1182 (2020). Lower Treg percentages have been observed with specific adverse pregnancy outcomes, however, existing literature on the role of Tregs in GDM

is conflicting. Several studies have assessed Treg percentages in GDM patients relative to healthy controls with conflicting conclusions. A meta-analysis of seven publications concludes that Tregs are significantly lower in women with GDM however, the mechanism of this association remains unclear. Functional assays suggest that Tregs in GDM patients may inefficiently regulate immune responses. For example, GDM Tregs are less effective at suppressing IFN-γ and TNF-α production in effector T cells and the activity of CD4 + T cells. Transcriptional networks may therefore be altered in Tregs as a result of GDM.

There is a distinct T-cell immune profile (Th1/Th2/Th17/Tregs) that undergoes fluctuations at different stages of gestation. This profile includes increased levels of Th1 cells and Tregs to accommodate the needs of the semi-allogeneic fetus, ensuring tolerance and preventing rejection. Towards the end of pregnancy, the immune system adjusts to facilitate labor initiation, leading to a decrease in Tregs.

However, in GDM, characterized by glucose intolerance and systemic inflammation, the immune response deviates from the norm observed in healthy pregnancies. Instead of the expected upregulation of T cells, there is a downregulation that may hinder the immune system's ability to counter proinflammatory factors like Th1 and Th17 cells. Research on the role of T-cells in GDM is varied and lacks consistency in design, contributing to the current mixed understanding of this aspect. This review critically examines existing research to enhance comprehension of the T-cell immune profile in GDM.

II. PERSONAL CONTRIBUTIONS

FIRST STUDY

5. General hypothesis and objectives

The incomplete understanding of GDM prevents the development of preventative treatment or a cure. Recent evidence published suggests that dysregulation of Regulatory T cells (Tregs) can cause GDM. However, the results conflict with one another. Therefore, we aimed to investigate the role of Tregs in GDM and optimise the protocol to isolate a high number of peripheral blood mononuclear cells (PBMCs) from pregnant women blood samples. To optimise PBMC isolation the frequency of PBMCs obtained after manually isolating or using SepMate tubes, and diluting blood in PBS or PBS containing 1mM EDTA was compared. Combining the addition of EDTA to blood dilution with SepMate tubes increased the number of viable T cells without significantly increasing overall PBMC frequency isolated. Flow cytometry was used to investigate Tregs in Healthy and GDM participants on a diet. We found two distinct groups of Healthy participants characterised by differences in memory CD4+, CD8+ T cells and CCR7+ and CD150+ Tregs. GDM participants showed a significantly lower percentage of naïve CD4+ and CD8+ T cells and higher TEMRA CD4+ T cells than Healthy (Alternative) participants, but no difference to Healthy participants. To conclude, Naïve and TEMRA cells and not Tregs were altered by the presence of GDM in participants on a diet. Thus, healthy participants can show distinct responses that affect the outcome of comparisons. However, further research is required to understand why this occurred.

We hypothesized that the frequency of Tregs from pregnant women who developed GDM and are on a diet are significantly lower in comparison to Healthy pregnant individuals.

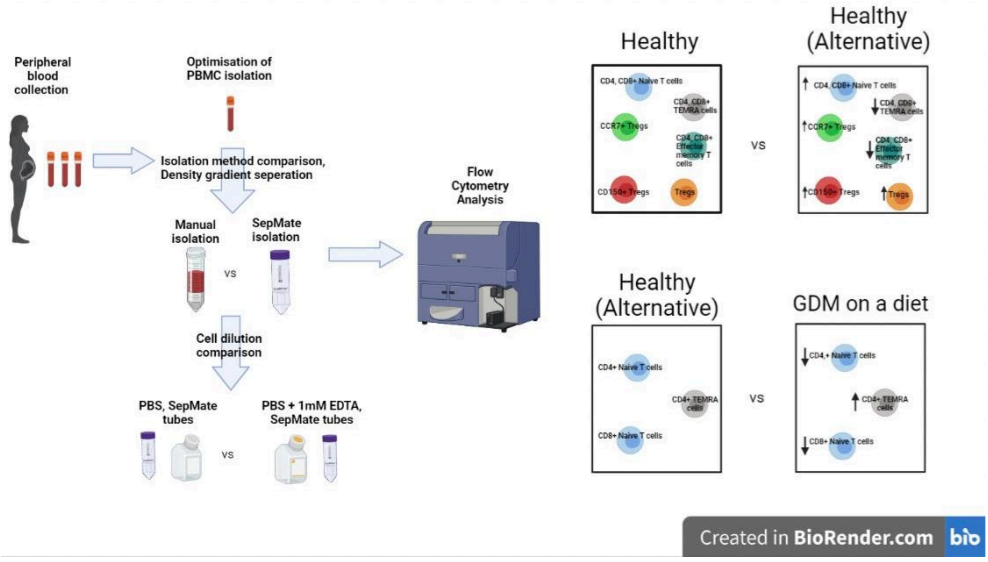

Figure 2. Flow Cytometry Analysis

The objectives of the study are:

- To optimise isolation of peripheral blood mononuclear cells (PBMCs), ensuring for a high frequency of T cells from whole blood samples obtained from pregnant women (cell count).
- Characterise T_{regs} and other T cell populations in PBMCs samples taken from Healthy pregnant or pregnant women with Gestational Diabetes Mellitus on a diet (Flow Cytometry).
- To investigate differences within the Healthy participants, identifying if the differences for CD4 T cells when using expression of CD45RA and CCR7 are significant enough to warrant splitting of Healthy participants between two groups (Flow Cytometry).
- Characterise T_{regs}, CD4+ and CD8+ T cells, comparing the two groups of Healthy participants identified and pregnant women with GDM on a diet (Flow Cytometry).
- To investigate if underlying demographic differences contribute to differences between Healthy participants (Flow Cytometry).

6. Methods. Protocol

The prospective study is to analyze immune cell populations accurately, a large number of Peripheral Blood Mononuclear Cells (PBMCs) must be obtained from blood samples. Traditional manual isolation and SepMate™ isolation of PBMCs consistently yield blood-stained plasma layers and overall low numbers of CD4+ and CD8+ cells. Here, we describe an optimized protocol, using PBS with EDTA to increase PBMC yield from pregnant patients. This protocol enables analysis of CD4+, CD8+, and Regulatory T Cells and is potentially applicable to any immune cell population.

Optimisation of PBMC isolation from whole blood samples was completed over two trials. The first trial compared between manual isolation of PBMCs (Manual Protocol) or the use of SepMate PBMC Isolation tubes (StemCell Technologies).

The protocol below describes the specific steps for isolating and freezing PBMCs from pregnant patients. The challenge of the procoagulant state of pregnancy (Sanches et al., 2020) (including an increase in clotting factors, reduced anticoagulant and fibrinolytic activity) made density centrifugation difficult, as the plasma layer was stained with blood. We found that use of EDTA in PBS to the blood before centrifugation improved separation and yield. Though blood is collected in EDTA lined tubes as standard, without dilution with further EDTA, the

plasma layer remained stained. This protocol was developed to analyze CD4+, CD8+ and Regulatory T Cell (Treg) populations, though this protocol may be useful for the analysis of any immune cell line in pregnancy.

6.1 Recruitment of pregnant women (patients and blood collection)

Timing: 10–15 min per sample

- 1. Collect venous blood in BD Vacutainer® EDTA Tubes (10 mL)
  a. Measure the usable whole blood volume to the nearest 0.5 mL
- 2. Keep the blood tubes at room temperature (18°C–22°C) under gentle agitation using an orbital shaker (90–100 rpm) until processing, as this improves the viability of PBMCs obtained. The blood sample should be processed as soon as possible after drawing, ideally within 8–12 h.

CRITICAL: Patient samples should be collected in accordance with international review board rules, including appropriate recruitment and patient consent. This study was approved by the King's College Hospital Research Ethics Committee, REC number is 02-03-033, dated 01/04/2003. All the experiments conform to the relevant regulatory standards. A total of 18 mL of blood in 2 × 9 ml EDTA tubes were taken from pregnant women at 35–36 weeks of gestation.

6.2 General preparation

- 3. Keep Ficoll-Paque PLUS (GE Healthcare Pharmacia, Cat No: 17144003) at room temperature (18°C–22°C)
- 4. Bring the PBS/EDTA with 2% FBS to room temperature (18°C–22°C)

Table 1. Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
18 mL of blood in EDTA tubes	Pregnant Women, 36 weeks, average age 32 years	n/a
Chemicals, peptides, and recombinant proteins		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ficoll-Paque PLUS	GE Healthcare Pharmacia	Cat No: 17144003
Phosphate Buffer Saline (PBS) with 1 mM EDTA	Lonza	Cat No: BE02-017F
Fetal Bovine Serum, qualified, heat-inactivated,	Gibco™, Thermo Fisher Scientific	Cat No: 26140087
Cell Freezing Medium-DMSO	Sigma-Aldrich	Cat No: 6164
RPMI 1640 W/O L-GLUTAMINE	LONZA	Cat No: 733-1690
Corning CoolCell	CORNING	CAT No: CLS432001-1EA
Cryovial, 2 mL, external thread, natural cap, 14 × 14 predefined datamatrix code, linear barcode and human readable code	Greiner Bio-One Ltd.	126263-2DG
CountBright Absolute Counting Beads	Thermo Fisher Scientific	Cat No: C36950
Software and algorithms		
BD FACSDiva™ Software	BD Biosciences	BD FACSDiva™ Software
ELabNext Software	Elab Inventory	ElabNext Eppendorf Group
Other		
SepMate™ 50 mL Tubes	Stem Cell Technologies	Cat No:154500
BD Vacutainer® EDTA Tubes (10 mL)	Fisher Scientific Ltd	Cat No: 10331254
Falcon 50 mL Conical Centrifuge Tubes	Thermo Fisher Scientific	Cat No:10788561

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Falcon 15 mL Conical Centrifuge Tubes	Thermo Fisher Scientific	Cat No: 11507411
BD LSRFortessa™ System	BD Biosciences	BD LSRFortessa™ System

6.3 Materials and equipment

PBS/EDTA with 2% FBS: PBS with 1 mM EDTA (Lonza Cat No: BE02-017F)) + 2% FBS (Thermo Fisher Scientific Cat No: 26140087). 10 mL of FBS in 500 mL PBS/EDTA. When FBS is added, keep it at 4°C, for a maximum of 5 days and use it at room temperature.

Table 2. Reagent and final concentration

Reagent	Final concentration	Amount
PBS/EDTA	PBS with 1 mM EDTA	490 mL
FBS	2%	10 mL

Cell Freezing Medium-DMSO: (Sigma Aldrich Cat No: 6164) to be stored in −20°C and at +4°C when open (keep on the ice during isolation)

Corning CoolCell Freezing 1°C/min cryo-freezing container (can hold 12 cryovials) (CAT No: CLS432001-1EA)

Alternatives: The SepMate™- 50 (Stemcell Technologies, Cat No: 154500) tubes were used for efficiency and time saving, though we found that their use did not significantly increase the number of PBMCs isolated. Manual separation, whereby layering the diluted blood and removal of the buffy coat is done by hand, may be performed. Proper technique is required to prevent contamination during layering and avoid disturbing the gradient when manually isolating the buffy coat layer after centrifugation.

6.4 Step-by-step method details

Isolation of PBMCs from peripheral blood

Timing: 1 h

This step describes how to isolate PBMCs from whole blood using SepMate™ Tubes (Blood et al., 2016)

1. Invert Ficoll-Paque PLUS several times.
2. Place 17 mL Ficoll-Paque PLUS medium into the SepMate™-50 tube by carefully pipetting it through the central hole of the SepMate™-50 insert (Ficoll to be just above the insert).
3. Transfer the blood to a separate 50 mL Falcon tube in a sterile manner, i.e., working in the hood, using sterile pipetting technique.
4. Dilute the blood 1:1 in PBS-EDTA with 2% FBS, close the tube and mix by careful inversion
   a. (In our study): add 16.5 mL blood +16.5 mL PBS-EDTA 2% FBS to reach a total of 33) mL; if less than 16.5 mL of blood is available, you can mix it with PBS-EDTA in a 1:1 ratio
   b. SepMate™-50 is designed to process 4–17 mL of an initial blood sample.
5. Keeping the SepMate™ tube vertical, add the diluted sample by pipetting it down the side of the tube. The sample will mix with the density gradient medium above the insert. *Take care not to pipette the diluted sample directly through the central hole.*
6. Centrifuge at 1200×*g* for 20 min at room temperature, with the brake on 7 (out of 9).

Note: Each tube contains four layers after the centrifugation, the yellow plasma layer on the top, the white MNC (mononuclear cells) layer, the Ficoll layer, and the red cell layer on the bottom.

7. Remove some of the plasma layer without removing the interface with the PBMCs, to allow better washing of the cells.
8. Pour off the top layer, which contains the enriched MNCs, into a new 50 mL Falcon tube. Do not hold the SepMate™ tube in the inverted position for more than 2 s as this may disrupt the filter within the SepMate™ tube, which ensures the Ficoll and red cell layer stay at the bottom.
9. Register the cryovials using the barcode and record them as PBMCs.
10. Fill up the 50 mL falcon tube with RPMI 1640 medium (Thermo Fisher Scientific, Cat No: 733-1690) and carefully mix.
11. Centrifuge at 300×*g* at 20°C for 8 min and discard the supernatant.
12. Repeat steps 10 and 11 using 5–10 mL PBS-EDTA with 2% FBS.

13. Centrifuge for 8 min at 300×*g* at 20°C and remove as much of the PBS-EDTA with 2%FBS as possible by pouring quickly without disturbing the pellet.

6.5 Freezing of PBMCs

Timing: ~ 24 h

Cryopreservation of PBMCs is vital for ensuring the integrity of the cells remains high after thawing, supporting high cell viability and ensuring sample data is representative of the donors.

14. Count and resuspend the cells in 1 mL of freezing medium DMSO: (Sigma Aldrich Cat No: 6164).
15. Ensure the cryovials (Greiner Bio-One Ltd Cat No: 126263-2DG) are resting on ice or in an icebox.
16. Aliquot cells into one barcoded cryovial, holding it on wet ice until freezing begins (within 5 min).
17. Freeze cells in the Corning CoolCell chamber (Cat No: CLS432001-1EA) below –70°C.
18. After at least 12 h, transfer the cells in cryo boxes and record their position.
19. Leave the cryo boxes in a −80°C freezer for 12–24 h (max four days), then transfer the cryovials into the liquid nitrogen tank.

7. Expected outcomes

The described protocol provides a reproducible method for isolating PBMCs, including lymphocytes (T cells, B cells, and Natural Killer cells), monocytes, and dendritic cells in pregnant patients. After thawing, the number of PBMCs obtained were counted using CountBright Absolute Counting Beads (Thermo Fisher Scientific, C36950) as per the manufacturer's instructions, using the BD Fortessa cell analyzer (BD Biosciences) combined with the BD FACSDiva software (BD Biosciences). Our protocol yielded an average of 53×10^6 PBMCs per healthy pregnant patient, though there will be biological variability in each individual.

Protocols for isolation of PBMCs have been optimized in non-pregnant patients, and there is no current standard method for isolation of PBMCs in pregnant patients. The standard protocol of dilution of collected blood with PBS alone yielded low numbers of CD4+ and CD8+ T cells in our pregnant population. We noted that after density centrifugation, the plasma layer

was blood-stained indicating potential contamination of the PBMCs. This can be seen in Figure 3, which demonstrates a comparison of diluted blood samples with PBS without EDTA (Figure 3A) and the addition of 1 mM of EDTA (Figure 3B). We considered the differences in haemostatic profile in pregnant women vs healthy controls – during pregnancy, the significant increase in clotting factors, reduced quantity of anticoagulants and reduced fibrinolytic activity creates a state of hypercoagulability (Sanches et al., 2020). This likely makes density centrifugation more complicated, and why the anticoagulant EDTA helped reduce blood contamination. The PBMCs obtained were counted using CountBright Absolute Counting beads and compared between the two conditions. In our case, the addition of EDTA to PBS increased the number of viable PBMCs obtained by an average of 15×10^6 cells (PBS: $38 \times 10^6 \pm 16 \times 10^6$, EDTA + PBS:$53 \times 10^6 \pm 8 \times 10^6$ cells) (Figure 4). The difference was not significant (p=0.2208), though upon analysis with flow cytometry, the addition of EDTA to PBS allowed a higher frequency of CD4+ T cells and CD8+ T cells to be isolated compared to PBS alone (Figure 5).

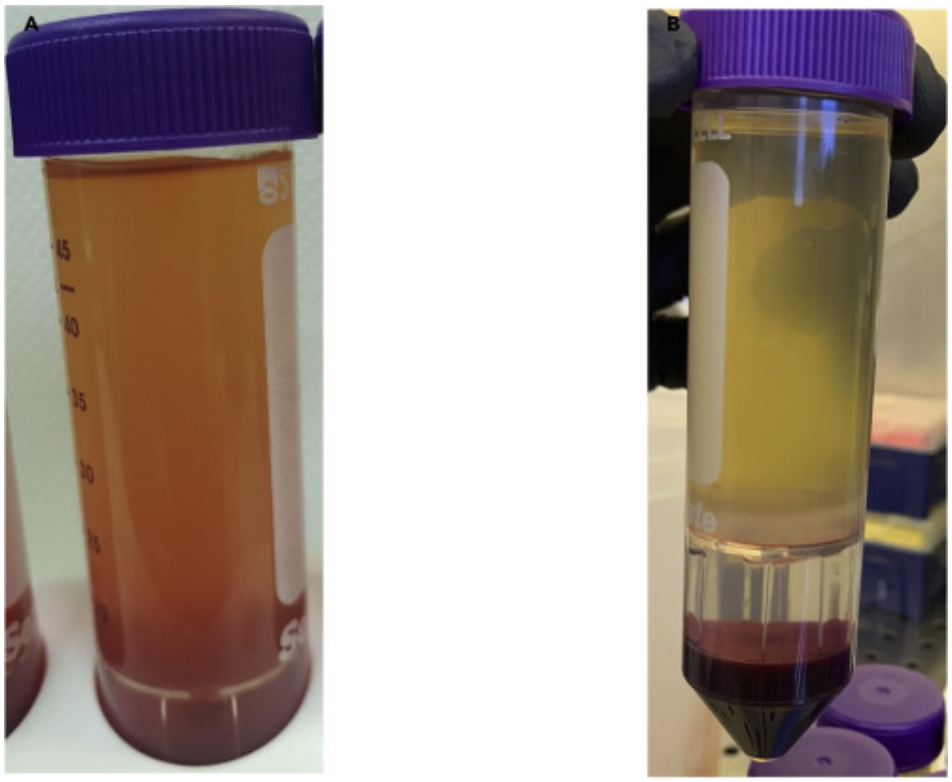

Figure 3. Comparison of cell separation using PBS and PBS EDTA
Compared to diluting blood samples in PBS without EDTA (**A**), the addition of PBS/EDTA 1 mM removed the blood staining the plasma layer (**B**)

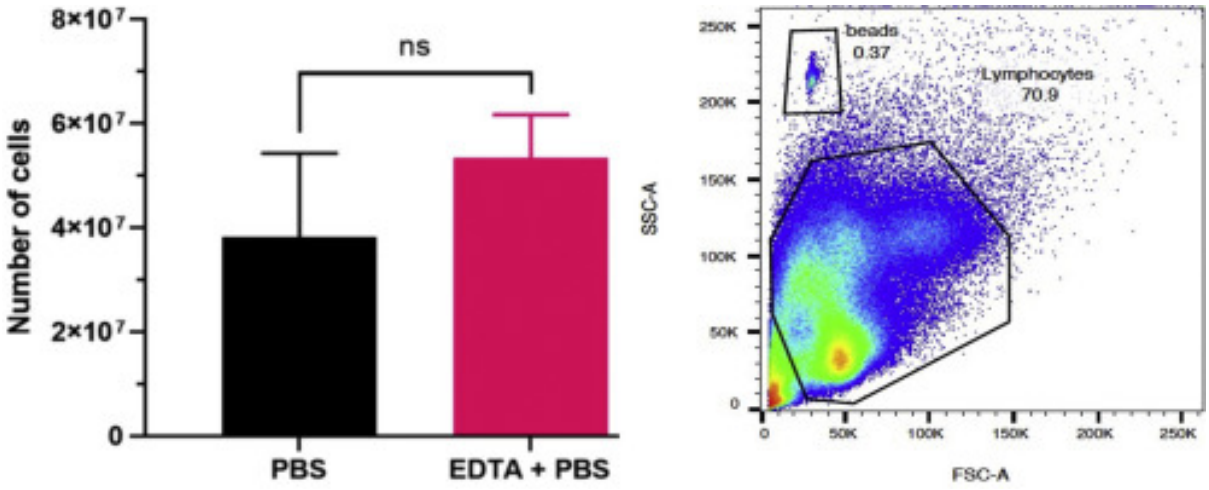

Figure 4. Flow Cytometric Analysis of T cells using PBS and PBS/EDTA 1 mM
Left panel: PBMCs were isolated from whole blood samples taken from healthy pregnant volunteers. Blood was diluted in either sterile PBS (PBS) or PBS containing 1 mM EDTA (PBS + EDTA). Addition of EDTA to PBS increased the number of viable PBMCs obtained by an average of 15 × 10^6 cells (Mean± Standard error of the mean, PBS: 38 × 10^6 ± 16 × 10^6, EDTA + PBS:53 × 10^6 ± 8 × 10^6 cells) Density centrifugation was completed using the SepMate™ tubes. The number of PBMC obtained were counted using the CountBright Absolute beads (right panel) and compared between the two conditions

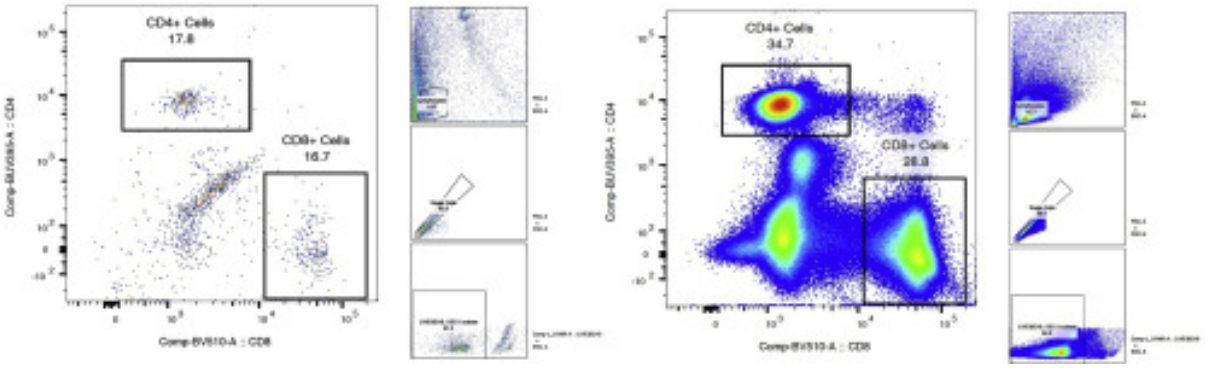

Figure 5. PBMCs were isolated from whole blood samples taken from healthy pregnant volunteers

8. Troubleshooting

Problem 1

Incomplete separation of whole blood into layers (step 6).

Potential solution

Ensure whole blood is fresh and uncoagulated (use of EDTA tubes, appropriate agitation, and storage at room temperature)

Reduce ejection speed of pipette and tilt SepMate™ tube. This will help to layer the whole blood onto the Ficoll slowly.

Problem 2

Decreased viability after freezing the cells (step 14)

Potential solution

When you cryopreserve cells, make sure that you work quickly and efficiently

Always work on ice and avoid leaving the cells in the freezing medium at room temperature after re-suspension.

Always use cryopreserve cells at −1°C/min using the Corning CoolCell chamber (Cat No: CLS432001-1EA).

Problem 3

Decreased viability after thawing the cells (when using own protocol)

Potential solution

When thawing, make sure that you work fast and efficient.

Submerge the cryovial with the PBMCs vial in a 37°C water bath for about 1 min.

Using the medium of your choice, add 1 mL of warmed (37°C) medium in the cryovial with the thawed PBMCs using a transfer pipette.

The thawed PBMCs can be poured in a 15 mL conical Falcon™ tube (catalog number 11507411) with 5 mL of warmed (37°C) medium.

Rinse the cryovials once with 1 mL of medium.

Incubate for 5 min, in the 15 mL conical Falcon™ tube (catalog number 11507411), in the 37°C water bath.

Centrifuge for 5 min at 300×*g* at room temperature.

Pour off the supernatant and proceed to further downstream applications.

SECOND STUDY

9. Methods and protocol

The following changes were made in the second trial. 16.6mL of blood was diluted in sterile DPBS or DPBS containing 1mM of Ultrapure 0.5M Ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Ref.15575-038). Other changes to the protocol including changing all washing stages to 300xg for 8 minutes. Samples were resuspended in 1mL of recovery cell freezing medium DMSO. Counting of cells during the second experiment was performed using the CountBright Absolute Counting Beads (Thermo Fisher Scientific, C36950) as per the manufacturer's instructions, using the BD Fortessa cell analyser (BD Biosciences) combined with the BD FACSDiva software (BD Biosciences). Data produced during the experiments was analysed using the FlowJo version 10.7.2 software (Tree Star) to create scatter plots and record cell events of the counting beads and live lymphocytes.

Blood samples were obtained from consenting healthy pregnant and pregnant women with GDM. Blood samples were stored for 10 hours at RT on an orbital shaker (Grant-Bio) before completed the PBMC isolation. The protocol used is as described in previous section, with the samples being diluted 1:1 in DPBS containing 1mM of EDTA (PBS-EDTA, Lonza BE02-017F) before density centrifugation in SepMate tubes. Samples were frozen in the Corning CoolCell chamber and later transferred into a liquid nitrogen tank for storage before being analysed.

a. Flow cytometry

Flow cytometry was used to characterise T regs and investigate differences between pregnant women with or without GDM.

A concise summary of the protocol for preparing and analyzing frozen PBMC samples using flow cytometry is shown below:

1. Thawing and Initial Preparation:
   - Thaw frozen PBMC samples in a 37°C water bath for 1 minute.
   - Transfer to falcon tubes with 8 mL of warm complete RPMI medium and incubate for 5 minutes at 37°C.
2. Centrifugation:
   - Centrifuge at 300xg for 10 minutes at room temperature, discard supernatant, and resuspend in 1 mL of complete RPMI.

3. Blocking:
    - Incubate in FCS buffer with a blocking reagent for 10 minutes to prevent non-specific binding.
4. Viability Staining:
    - Wash cells and incubate with Fixable Viability Dye (FVD) eFluor® 780 for 30 minutes at 2-8°C, then wash again.
5. Surface Marker Staining:
    - Incubate with an antibody cocktail for surface markers for 30 minutes at 4°C, followed by a wash.
6. Intracellular Staining:
    - Fix cells with IC Fixation Buffer, permeabilize, and then incubate with an intracellular antibody cocktail for 30 minutes at room temperature.

7. Data Acquisition: was analysed using the FlowJo software to record percentages of the lymphocytes to identify populations that express the target receptors and intracellular molecules. The data were then exported and analysed using the GraphPad Prism software (Version 9, GraphPad Software).

b. Final Conclusions and future perspectives

Our study demonstrated that there could be differences between Healthy pregnant women that can affect the outcome of comparisons to pathological conditions. This is something that in important to consider when designing and completing experiments to study responses during pregnancy. Especially when studying populations of memory T cells and Tregs where we showed that there were significant differences between the two groups of Healthy pregnant women.

We were not able to find a cause for why these differences existed. Therefore, our results support further investigation into these populations, including CCR7 or CD150+ Tregs and TEMRA cells which have not been well researched for their involvement in pregnancy. Investigations into this could be completed in vitro first to investigate effect on other immune cell populations. This could include using suppression assays to compare the addition of CCR7+ or CD150+ Tregs to cells taken from decidua or cord blood and comparing them to the negative control. We could also look at the differences in cytokines produced by these cells using an enzyme-linked immunosorbent assay. We could also subset these cells in human or

animal pregnancy and use transcriptomics to investigate how these cells differ to other Tregs and conventional T cells.

The current method using flow cytometry also only allowed for analysis of cells using up to 18 markers. Mass spectrometry could improve on this as it allows analysis for expression of 33 markers. We could use mass spectrometry to investigate the link with other populations of immune cells, allowing us to find if what we have found in our results is also reflected in other immune cell populations. We could also use it to identify if more patients exhibit the same response as the Healthy (Alternative) participants to prove if the results are replicable or not. To look deeper at specific populations and functional changes caused by GDM we could also use a 10X genomic as it may be more sensitive to picking up changes that flow cytometry couldn't detect and we look at functional changes we could not investigate otherwise.

Further evaluation into the effect of diet on Tregs and how diet mediate the changes is suggested due to the differences in our results to previous findings. By comparing between different diets, including in composition of carbohydrates, fats and protein, we may be able to better understand how diet effects immune cell responses and which parts of a persons' diet the best are to target. This could potentially become a form of treatment that indirectly regulates the immune system without causing harsh side effects. It could also be potentially implemented alongside formal treatments in the future to help improve the efficacy of the treatment by indirectly reducing or improving immune responses in patients where they are not responding well.

To conclude, the combination of diluting blood in PBS containing 1mM EDTA and performing density centrifugation in SepMate tubes, increases the viability of T cells within PBMCs for analysis using flow cytometry by removing granulocyte contamination. While we do not know why we had two groups of Healthy participants it could be suggested to relate to how near the mother is to giving birth. This is an area that could be researched and correlated with levels of memory T cells or Tregs and could help to improve our understanding of the mechanism of labor better. Finally, diet can potentially reduce the effect of GDM by indirectly increasing the frequency of Tregs. However, this is only a suggestion and further research is needed to study the potential of using changes in diet to treat or prevent GDM.

THIRD STUDY

10. Aims and objectives

This study aimed to investigate the Treg transcriptional landscape in GDM through scRNA-seq. We identified genes differentially expressed within Treg subsets from GDM patients and detected gene sets altered in GDM Tregs. We explore the potential for these cell-type-specific signals to serve as clinical biomarkers by modelling gene expression and GDM status across cohorts. This work provides a compendium of dysregulated genes within FOXP3 + Tregs and expands our understanding of their contribution to the pathophysiology of GDM.

11. Methods

Regulatory T cell dysfunction is proposed to underlie the pathology of gestational diabetes mellitus (GDM). We present single-cell transcriptomes of Tregs and CD4 + T cells isolated from PBMCs of women with GDM and healthy pregnant women. Our analysis identifies naive and effector molecular subsets in Tregs with a significant increase in memory CD4 + T cells from GDM patients, suggesting impaired immune suppression. Differential expression analysis revealed a decrease in AP-1 transcription factor subunits in the Naive-2 cluster, and pathway analysis indicated downregulated NF-kB signalling. Additionally, Effector-2 Tregs upregulated genes involved in Angiogenesis. We evaluated the translational potential of scRNA-seq-derived expression markers from CD4 + T cell populations and validated their predictive potential in pseudobulk CD4 + T cells and independent validation cohorts. The percentage of Tregs in GDM patients is observed to be reduced in women with GDM (Szabo, P. A. *et al.* Single-cell transcriptomics of human T cells reveals tissue and activation signatures in health and disease). We identified FOXP3 + cells within sorted CD4 + T cells which we expect to contain Tregs, however, their proportion did not significantly differ between conditions. FOXP3 + cells represent a low proportion of the CD4 + T cell pool. Therefore, we may be underpowered to observe the effect at a single cell level. Of note, we observed Humanin + and *MALAT1* + cell clusters in both Treg and CD4 + T cell populations. These cells have been previously reported in sorted Tregs from ankylosing spondylitis patients profiled with the 10X scRNA-seq platform (Simone, D. *et al.* Single cell analysis of spondyloarthritis regulatory T cells identifies distinct synovial gene expression patterns and clonal fates). We also observed Humanin + CD4 + T cells, have been reported in the context of Rheumatoid Arthritis (Argyriou, A. *et al.* Single cell sequencing reveals expanded cytotoxic

CD4+ T cells and two states of peripheral helper T cells in synovial fluid of ACPA+ RA patients). To our knowledge, this is the first report of Humanin-positive cells in GDM. Altered humanin concentrations have been proposed as a biomarker for diabetic conditions, as Humanin is protective under oxidative stress, which can arise from excess ROS generation from diabetic hyperglycaemia (Boutari, C., Pappas, P. D., Theodoridis, T. D. & Vavilis, D. Humanin and diabetes mellitus: A review of in vitro and in vivo studies.), Rochette, L., Meloux, A., Zeller, M., Cottin, Y. & Vergely, C. Role of humanin, a mitochondrial-derived peptide, in cardiovascular disorders.

Further research into the phenotypes of these Treg subsets is required to understand their role in GDM.

12. Results and conclusions

While our study sheds light on the Treg transcriptional landscape in GDM, the focus on isolated CD4 + cells carries limitations. Several immune subtypes that were not studied are associated with the pathophysiology of GDM, such as Neutrophils, Macrophages and Monocytes (48). Functional studies in models of GDM may further uncover the role of immune cells in attenuating Treg activity. For example, we observed the under-expression of *JUNB*, the AP-1 transcription factor, which is demonstrated to regulate intestinal Treg development and its ablation is associated with increased T helper accumulation and inflammation Wheaton, J. D. & Ciofani, M. JunB Controls Intestinal Effector Programs in Regulatory T Cells.

Although single-cell RNA-seq empowers us to discover cell type-specific expression, this analysis remains limited to protein-coding RNAs. In contrast, several non-coding RNAs such as microRNAs (Fan, W., Pang, H., Xie, Z., Huang, G. & Zhou, Z. Circular RNAs in diabetes mellitus and its complications and circular RNAs (Guarino, E. *et al.* Circulating MicroRNAs as Biomarkers of Gestational Diabetes Mellitus: Updates and Perspectives, have been proposed as candidate circulating biomarkers for GDM. Studying the effect of non-coding RNAs on T regulatory cell subtypes may uncover insights into GDM pathology.

In conclusion, this study identifies Treg subsets with altered transcriptional programmes in GDM patients and proposes candidate marker genes with translational potential that arise from Treg dysregulation. Chronic inflammation leading to metabolic dysregulation is one coupling factor driving the association between obesity, cardiovascular disease, and diabetes (Hotamisligil, G. S. Inflammation, metaflammation and immunometabolic disorders).

Future work investigating these molecular markers of Treg dysfunction will improve our understanding of the complex immunometabolic state observed in GDM.

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List of scientific papers published

1. Isolation and freezing of human peripheral blood mononuclear cells in pregnant patients - protocol paper *published STAR PROTOCOLS Volume 3, Issue 1, 18 March 2022, 101204- within the thesis the information can be found in chapter 2, pages 40-50* Athina Efthymiou,[1]* **Nicoleta Mureanu**,[1*] Rebecca Pemberton[2], Sarah Tai-MacArthur[2], Daniela Mastronicola[2], Cristiano Scotta[2], Giovanna Lombardi[2], Kypros H Nicolaides[1] and Panicos Shangaris[1,2.] *shared co-authors. DOI: 10.1016/j.xpro.2022.101204
2. Regulatory T Cells in Pregnancy adverse Outcomes - (Rutepo): A Systematic Review & Meta-Analysis published in *Frontiers Immunology Editorial Office Oct 2021* Samantha GREEN[1*], Marina POLITIS[2*], Kathrine S RALLIS[3*], Alba Saenz de Villaverde CORTABARRIA[4], Athina EFTHYMIOU[5], **Nicoleta MUREANU[5]**, Kathryn DALRYMPLE[5], Cristiano SCOTTA[5], Giovanna LOMBARDIi[5], Rachel M TRIBE[5], Kypros H NICOLAIDES[5], Panicos SHANGARIS. DOI: 10.3389/fimmu.2021.737862
3. The role of T Cells in Gestational Diabetes and their therapeutic potential, a literature review
   Vienna Hubbard[1], Athina Efthymiou[2,3] ,**Nicoleta Mureanu[2,3]** , Ffion Harris[4], Daniela Mastronicola[4], Cristiano Scotta[4], Giovanna Lombardi[4], Timothy Tree[4], Kypros H Nicolaides[2,3] and Panicos Shangaris[2,4]
   Volume 14 - 2023 | https://doi.org/10.3389/fimmu.2023.1226617
4. Regulatory T cells (Tregs) in the peripheral blood of women with gestational diabetes; a systematic review and meta-analysis published in *Frontiers in Immunology December 2023*
   Hania Arain[1], Tina Patel[1], **Nicoleta Mureanu** [1,2] , Athina Efthymiou [1,2] , Giovanna Lombardi[3], Timothy Tree[3], Kypros H Nicolaides[1,2] and Panicos Shangaris[1,3] W*ithin the thesis the information can be found in chapter 2 , pages 37-38, respectively 107-108*
   DOI: 10.3389/fimmu.2023.1226617
5. Single-cell transcriptomics reveals markers of regulatory T cell dysfunction in Gestational Diabetes Mellitus *under review at Nature portofolio journal December 2023.*

Nana E. Mensah*, Athina Efthymiou*, **Nicoleta Mureanu***, Shichina Kannambath, Heli Vaikkinen, Amanda Bowman, Athul Menon, Tim Tree, Cristiano Scotta, Giovanna Lombardi, Pawan Dhami, Kypros H Nicolaides and Panicos Shangaris. *shared co-authors. *Within the thesis the information can be found in chapter 2, pages 90-107* DOI: 10.21203/rs.3.rs-3773991/v1

6. The immunomodulatory role of regulatory T cells in preterm birth and associated pregnancy outcomes

   Nicoleta Mureanu [1**], Amanda M. Bowman [1**], Imogen A. Porter-Wright [2**], Priya Verma[**], Athina Efthymiou, Kypros H. Nicolaides, Cristiano Scotta, Giovanna Lombardi**, Rachel M. Tribe**, Panicos Shangaris** - in process of being published (accepted by Nature portofolio journal)