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Dynamic changes in neutrophil activation and metabolic profile during pregnancy are associated with gingival inflammation

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Neutrophils are key players in both periodontal and placental immunity, undergoing profound immunometabolic and functional changes during pregnancy. Their excessive activation has been linked to gestational complications. Gingivitis, and even more so periodontitis, the advanced stage of this condition, are chronic inflammatory diseases that frequently worsen during pregnancy and have been associated with adverse outcomes such as fetal growth restriction and placental dysfunction. Although bacterial dissemination and inflammation are thought to mediate this link, the underlying mechanisms remain poorly understood. How neutrophil activation and metabolism evolve throughout pregnancy—and how this relates to the exacerbation of periodontal inflammation—remains largely unexplored. We analyzed the immunometabolic and activation profiles of circulating neutrophils from pregnant women at 16–20 weeks and term, alongside their gingival inflammatory status. Our findings show that pregnancy reprograms neutrophil metabolism, promoting a progressive shift towards enhanced glucose utilization and increased lipid droplet accumulation at term. Basal reactive oxygen species production increased throughout pregnancy and correlated with gingival inflammation. Basal and PMA-induced neutrophil extracellular trap release also increased with gestation. Gingival crevicular fluid samples further stimulated neutrophil activation, particularly those enriched in *P. gingivalis*. These results reveal a dynamic immunometabolic rewiring in maternal circulating neutrophils throughout pregnancy, suggesting its modulatory role in the interplay between systemic immunity and oral inflammation.

Keywords Human neutrophils, Pregnancy, Immunometabolism, Periodontal disease

Periodontal disease is a chronic inflammatory disease characterized by the destruction of the periodontal ligament and alveolar bone loss[1]. Gingival inflammation is a common feature during pregnancy, and it is associated with an increased risk of pregnancy complications and reduced fetal growth[2–5]. Mechanistic studies provide strong evidence that periodontal pathogens can translocate from the infected periodontium to the feto-placental unit, triggering a detrimental inflammatory response[2,6]. In turn, bacterial extracellular vesicles from periodontal pathogens can be released and transported to distant vascularized tissues, including the placenta[7,8]. However, the biological mechanisms connecting inflammation in the periodontium with placental deficiency and fetal growth restriction remain unclear.

Neutrophils play a key role during normal pregnancy, regulating immune tolerance to fetal antigens and contributing to vascular transformation at placentation[9,10]. At the same time, excessive activation of neutrophils

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affects pregnancy outcome[10]: neutrophil activation with neutrophil extracellular trap (NET) production has been reported in recurrent fetal loss[11], preeclampsia[12] and gestational diabetes[13]. Cellular metabolism is increasingly recognized as a key determinant of immune cell phenotype and function. In neutrophils, metabolic adaptations and distinct regulations have been identified as critical for their differentiation and profiling[14]. However, evidence on their immunometabolic profiling at different stages of pregnancy is lacking, as well as the relationship between their activation profile and the worsening of gingival inflammation in pregnant women. Moreover, studies have primarily focused on the third trimester, with limited research exploring their profiles throughout the entire course of pregnancy[12,15–17].

The aim of this work was to deepen into the functional and metabolic reprogramming of circulating neutrophils throughout normal pregnancy and to explore possible associations with the periodontal status of these women. We characterized immunometabolic pathways and the activation profile of neutrophils in a population of women at mid pregnancy (16–20 weeks) and at term, along with a clinical periodontal evaluation of each patient. Nonpregnant women were studied in parallel as a control group. Results indicate that pregnancy entails a metabolic rewiring of maternal circulating neutrophils which become increasingly activated as pregnancy progresses. Neutrophils at normal term pregnancies presented an attenuated response towards inflammatory stimuli, thus limiting excessive activation. Neutrophil progressive activation during pregnancy correlated with the periodontal status of the women[2].

Materials and methods

This was a cross-sectional study comparing the level of gingival inflammation and circulating neutrophil profiles of 16–20-week pregnant women, term pregnant women and age-matched non-pregnant controls.

Study population

All participants were between 20 and 41 years old and were recruited from a public healthcare hospital in the city of Buenos Aires. For pregnant women, inclusion criteria were single pregnancy with gestational age confirmed by ultrasound, absence of systemic diseases, body mass index (BMI) between 18.5 and 30 (WHO guidelines) before pregnancy, and no periodontal treatment during pregnancy. Exclusion criteria were diagnosis of hypertension, preeclampsia, gestational diabetes, autoimmune disorders, infectious diseases (positive serology for syphilis, hepatitis B, Chagas disease, toxoplasmosis, HIV), presence of antiphospholipid antibodies, multiple pregnancy, smoking, antibiotic use within the previous three months, or recent dental treatment likely to alter gingival status. For women at term (≥ 37 weeks of gestation), only those who underwent planned caesarean section were selected and samples were collected at the time of admission or immediately after delivery. Maternal and neonatal parameters were evaluated at birth to support the classification of a normal pregnancy outcome. These included, but were not limited to, appropriate birth weight, normal Apgar score, gestational age consistent with term delivery (as assessed by Capurro method), and no admission to the neonatal intensive care unit (NICU).

Nonpregnant women were selected with similar social and epidemiological criteria and inclusion requirements were being systemically healthy, not pregnant in the last 12 months and not breastfeeding. Exclusion criteria were the same as for pregnant women, including BMI outside the 18.5–30 range, systemic diseases, infectious diseases, ongoing periodontal therapy, antibiotic use within the past three months, smoking, or recent dental treatment.

Table 1 shows clinical, periodontal and microbiological assessments of the three populations.

Blood samples

Peripheral blood samples were obtained from 25 healthy non-pregnant donors, 40 pregnant women at 16–20 weeks of gestation, and 30 women at term. For term women, blood samples were collected at the time of admission for delivery.

Human neutrophil isolation

As previously[9,17], neutrophils were isolated by centrifugation on Ficoll-Paque PLUS (GE Healthcare), Dextran sedimentation (MP Biomedicals) and hypotonic lysis. Neutrophils were then suspended in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Internegocios). The purity was routinely checked by flow cytometry (> 98%). Cells were used immediately after isolation.

Oral examination

Oral examination was performed under appropriate lighting conditions, using standardized instruments (North Carolina millimeter-type probe and oral mirror). Gingival inflammation status was evaluated in four sites per tooth, excluding third molars, and registered using Löe and Silness Gingival Index (GI) and the presence of biofilm was established using Sillness and Löe Plaque Index (PI). All examinations were conducted by two calibrated specialists in periodontology from the Department of Preventive and Community Dentistry, University of Buenos Aires, following a standardized calibration protocol prior to study initiation. Given that gingivitis is more prevalent than periodontitis in pregnant women, mainly due to the younger age of this population, and that performing a complete periodontal examination (including CAL and PD measurements) immediately before or after cesarean delivery is logistically difficult, the study focused on gingival indices rather than full periodontal diagnosis. This approach ensured consistent, standardized data collection across all participant groups. None of the women had aggressive periodontitis.

Crevicular fluid sampling

After removal of the supragingival biofilm with a curette, Gingival Crevicular Fluid (GCF) was collected through consecutive insertion of four sterile paper cones into the pocket for 20 s. To model active inflammation, we

	Patient groups			
	Non pregnant	16–20 weeks pregnant	Term	*p* value
Gestational age	na	17.5 ± 0.25	38.53 ± 0.09	na
Pregnancy outcome	na	Normal	Normal	na
Sample size	25	40	30	na
Clinical and microbiological parameters				
Gingival index (GI)	0.234 ± 0.078[a]	0.855 ± 0.060[b]	1.418 ± 0.099[c]	a: 0.0024 b: < 0.0001 c: < 0.0001
Plaque index (PI)	0.357 ± 0.118[a]	0.831 ± 0.045[b]	1.494 ± 0.088[c]	a: 0.005 b: < 0.0001 c: < 0.0001
% sites GI > 1	3.7 ± 1.7[a]	30.6 ± 2.3[b]	54.1 ± 4.6[c]	a: 0.0005 b: < 0.0001 c: 0.0002
% sites PI > 1	7.5 ± 2.2[a]	30.0 ± 2.0[b]	58.5 ± 4.3[c]	a: 0.0014 b: < 0.0001 c: < 0.0001
Bacterial load (16S copies/µl)	2.43E + 05 ± 7.70E + 04[a]	7.58E + 06 ± 2.11E + 06[b]	3.70E + 07 ± 1.21E + 07[c]	a: 0.0044 b: 0.0081 c: 0.0245
Positive samples Fn	100%	100%	100%	
Positive samples Pg	12.5%	57.1%	60%	

Table 1. Clinical, periodontal and microbiological assessments of populations. Values are mean ± SEM, Kruskal–Wallis followed by Dunn's multiple comparisons tests were performed. na: Not applicable.

selected the four sites with the highest GI per patient. The samples were placed and transported in microtubes with reduced transfer fluid (RTF medium) and stored at − 80 °C. Functional studies were carried out with diluted samples (1:5).

Microbiological studies in GCF samples

Genomic DNA was extracted from GCF samples using affinity columns (Presto TM Mini gDNA Bacteria kit, Geneaid) following the manufacturer's instructions. The relative quantification of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* was carried out by qPCR using DNA strand SYBR Green Master Mix (Bio-Rad) with specific primers (Pg: F: 5-t'ACGAATCAAAGGTGGCTAAGTT3t', R 5t'TTAGTCGCATTTTCGGCTGAT3t'; Fn: 5′ACCTAAGGGAGAAACAGAACCA3′, R: 5t'CCTGCCTTTAATTCATCTCCAT3t'). Determination of total bacteria was performed with oligonucleotides for a region of the 16S subunit (F: 5t'CGCTAGTAATCGTGGATCAGAATG3t', R: 5t'TGTGACGGGCGGTGTGTA3t')[18].

ROS production

Neutrophils were stimulated for 45 min and 5 µM 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) (Sigma-Aldrich) was added for another 15 min, then cells were thoroughly washed and analyzed by flow cytometry as before[19]. Depending on the experimental setting, as described in each figure, stimulation was performed using 80 nM PMA, 100 ng/mL LPS, or GCF.

Neutrophil migration

Neutrophils were pretreated with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), washed and placed on top of a 5 µm transwell. Cells that migrated after 60 min were collected and quantified using a flow cytometer.

2-NBDG glucose analogue uptake

Neutrophils were stimulated for 60 min with 80 nM PMA or 100 ng/ml LPS (Sigma-Aldrich), medium was removed, and cells were incubated with 100 µM 2-NBDG, a fluorescent glucose analog, (Thermo Fisher Scientific) and, after several washes, cells were analyzed by flow cytometry as previously[17,20]. Data were immediately acquired in a FACS Aria II cytometer (BD Biosciences) and analyzed using FlowJo™ software.

Lipid droplets quantification

Neutrophils were stimulated for 180 min using 80 nM PMA or 100 ng/mL LPS, washed twice with PBS and incubated with 2 µM BODIPY 493/503 (Thermo Fisher Scientific) for 15 min at 37 °C. They were then washed with cold PBS and resuspended in 2% FBS in PBS solution for immediate flow cytometry analysis.

Long-chain fatty acids (LCFA) uptake

Neutrophils were stimulated using 80 nM PMA or 100 ng/mL LPS and then incubated with BODIPY-FL C12 (Thermo Fisher Scientific) as previously[20]. Briefly, BODIPY-FL C12 is a 12-carbon chain length saturated fatty acid linked to the fluorophore BODIPY, resembling an 18-carbon fatty acid. The probe was preincubated with 0.1% fatty acid-free bovine serum albumin (Sigma-Aldrich) for 30 min at 37 °C. Cells were washed with PBS and incubated with 5 µM BODIPY-FL C12 solution in serum-free RPMI-1640 for 5 min. Cells were washed with

0.2% BSA, resuspended in 2% FBS in PBS solution, and data were acquired and analyzed as for glucose uptake assay.

Elastase activity and DNA determinations

Neutrophils were stimulated in phenol red-free medium with PMA at concentrations ranging from 0 to 80 nM, or with GCF, for a duration of 210 min. Cells were then treated with 1 U/ml DNase for an additional 30 min, and supernatants were collected[19,21]. DNA concentration was determined fluorometrically with 5 µM Sytox Green (Sigma-Aldrich) and neutrophil elastase activity was measured using 1 mM human granulocyte elastase substrate (Sigma-Aldrich).

Statistical analysis methods

Data was analyzed using GraphPad Prism 9.4 software. When comparing two groups, Wilcoxon matched pairs was used and for multiple comparisons, Friedman or Kruskal–Wallis test were used with corrected Dunn's test when applicable. Results are expressed as Mean ± SEM. Spearman correlation was used for association between variables.

Ethical considerations

Studies were approved by the Institutional Review Board and Ethics Committee of Argerich Hospital of Buenos Aires (DI201874-HGACA), the School of Dentistry of the University of Buenos Aires (#20211157) and the Clinical Investigation Society of Argentina (#10469/23). All donors provided written informed consent for sample collection and subsequent analysis. Moreover, all experiments were performed in accordance with the relevant guidelines and regulations.

Results

Metabolic reprogramming of circulating neutrophils during pregnancy

Given the extensive evidence highlighting the pivotal role of immunometabolic reprogramming in shaping immune cell fate and driving distinct functional phenotypes, we first investigated a potential metabolic rewiring in neutrophils throughout pregnancy. Neutrophils were isolated from different populations -non-pregnant (Non preg); 16–20 weeks pregnant (16–20w); term pregnant (Term)- and stimulated or not with PMA (Fig. 1 left panel) or LPS (Fig. 1 right panel), as two classical neutrophil activators. Regarding glucose metabolism, neutrophils highly increased the glucose analogue 2-NBDG uptake when stimulated with PMA or LPS compared to basal conditions, in the three cohorts (Fig. 1A). However, neutrophils from 16 to 20 weeks pregnant women were not as efficient to increase glucose uptake upon PMA-stimulation as observed in the non-pregnant counterpart: (1.85-fold increase versus 1.40, $p < 0.05$) (Fig. 1B). Additionally, neutrophils from women at term presented the highest increase in glucose uptake when stimulated compared to neutrophils from 16 to 20w pregnant women (2.1-fold increase versus 1.40 for PMA and 1.45-fold increase versus 1.12 for LPS, all $p < 0.05$) (Fig. 1B). Regarding lipid metabolism, neutrophils from women at term and from non-pregnant women accumulated more lipid droplets after stimulation (Fig. 1C), unlike 16-20w neutrophils. Finally, neutrophils from non-pregnant women increased the uptake of LCFA after activation with PMA or LPS as reported[17,22], whereas neutrophils during pregnancy did not (Fig. 1D).

ROS production and NET release increase throughout pregnancy

We next ought to explore the function and priming of neutrophils ex vivo in the three cohorts. Neutrophil ROS production increased progressively throughout pregnancy, reaching its highest levels at term (Fig. 2A). PMA and LPS increased ROS production in neutrophils from all three cohorts, but to varying degrees. The highest fold increase was observed in non-pregnant women after PMA stimulation (7.8-fold), followed by those at 16–20 weeks (3.6-fold) and at term (2.7-fold) (Fig. 2A, left). The same pattern can be observed with LPS stimulation (Fig. 2A, right). Figure 2B shows a representative dot plot of isolated neutrophils (left) and histogram of unstimulated and activated neutrophils (right). NET release was then assessed using PMA at concentrations ranging from 0 to 80 nM, a well-known NET inducer. Neutrophils from women at term released more NETs compared to the other two groups at all concentrations tested whereas 16–20w neutrophils released more NETs than neutrophils in the non-pregnant group (Fig. 2C). PMA stimulated NET release at all tested concentrations in the three groups (Supplementary Fig. 1). No differences were found in migration capability between groups, evaluated using a transwell assay (Fig. 2D).

Neutrophils' ROS response diminishes with gestational age and correlates with the gingival status of the women

Gingival inflammation worsens as pregnancy progresses, as quantitatively assessed by increases in all clinical parameters monitored throughout gestation (Gingival and Plaque Indices, % sites with GI or PI greater than 1) (Table 1). Additionally, gingival crevicular fluids obtained from these women also present a higher number of bacteria as pregnancy advances (Table 1).

Considering that gingival inflammation worsens and basal ROS production by neutrophils increases progressively throughout pregnancy (Figs. 2A and 3A), we next explored correlations between gingival inflammation and ROS production, using Spearman's correlation test. There is a positive correlation between gingival and plaque index with ROS generation in unstimulated neutrophils ex vivo (Fig. 3A). On the other hand, a negative correlation was observed between the PMA or LPS response (measured as fold increase relative to basal) and the clinical oral parameters (Fig. 3B–C). These results indicate that during normal pregnancy, neutrophils display higher basal ROS levels alongside a moderated proinflammatory response upon stimulation.

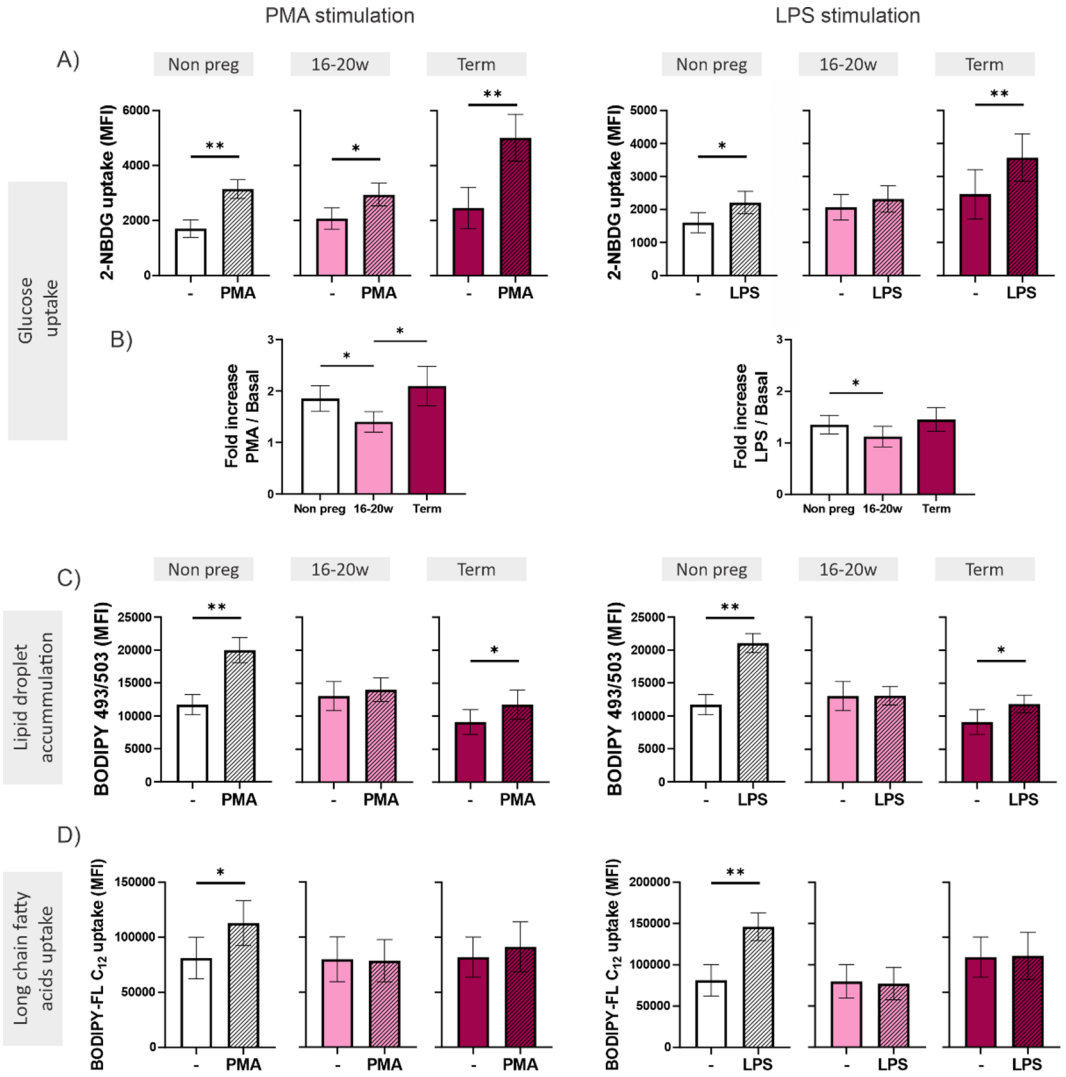

Fig. 1. Metabolic reprogramming of circulating neutrophils during pregnancy. Neutrophils from non-pregnant, 16–20-week pregnant and term women were obtained and stimulated with 80 nM PMA (left panel) or 100 ng/ml LPS (right panel). (**A**) 2-NBDG incorporation to assess glucose uptake, (**B**) Relative values from fluorescence intensity of 2-NBDG relative to their basal fluorescence are shown. (**C**) BODIPY 493/503 to stain lipid droplets and (**D**) BODIPY-FL C12 incorporation for long chain fatty acid uptake evaluation. They were evaluated by flow cytometry, where mean fluorescence intensity (MFI) was determined. Each graph displays data from at least 7 samples per population. Values are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, Wilcoxon matched pairs test was used in A, C and D whereas Friedman with corrected Dunn's test was used in B.

While the implications for pregnancy outcomes require further investigation, this pattern may reflect an adaptive immune modulation.

Gingival crevicular fluid activates neutrophils

Based on the observed correlation between neutrophil ROS production and gingival-associated indices, we next assessed the direct impact of components of the GCF taken from the site of infection at different states of gestation on neutrophil activation. We used an in vitro model to mimic the interaction between circulating neutrophils and the components of the inflamed gingival tissue. Neutrophils from control non-pregnant women were cultured in the presence of GCF from women of the three cohorts and ROS and NETs were next evaluated, as depicted in the illustration in Fig. 4A. GCF samples from women at term stimulated the highest ROS production, followed by those from 16-20w pregnant women (Fig. 4B). This increase was dose-dependent on all cohorts (Fig. 4C). Considering that *F. nucleatum* (Fn) and *P. gingivalis* (Pg) are common periodontal pathogens in pregnancy-associated gingivitis, their relative abundance in the GCF was tested in relation to the production of ROS by neutrophils of the three groups. The total number of bacteria found in GCF increases (Fig. 4D left, shown as 16S) as pregnancy progresses, as well as the % of *F. nucleatum* (Fig. 4D, center). The % of *P. gingivalis* (Pg) is higher in GCF from pregnant women, but no differences were found in 16–20w or term samples (Fig. 4D, right). ROS increase, assessed as MFI of GCF-stimulated condition divided by the basal condition, positively

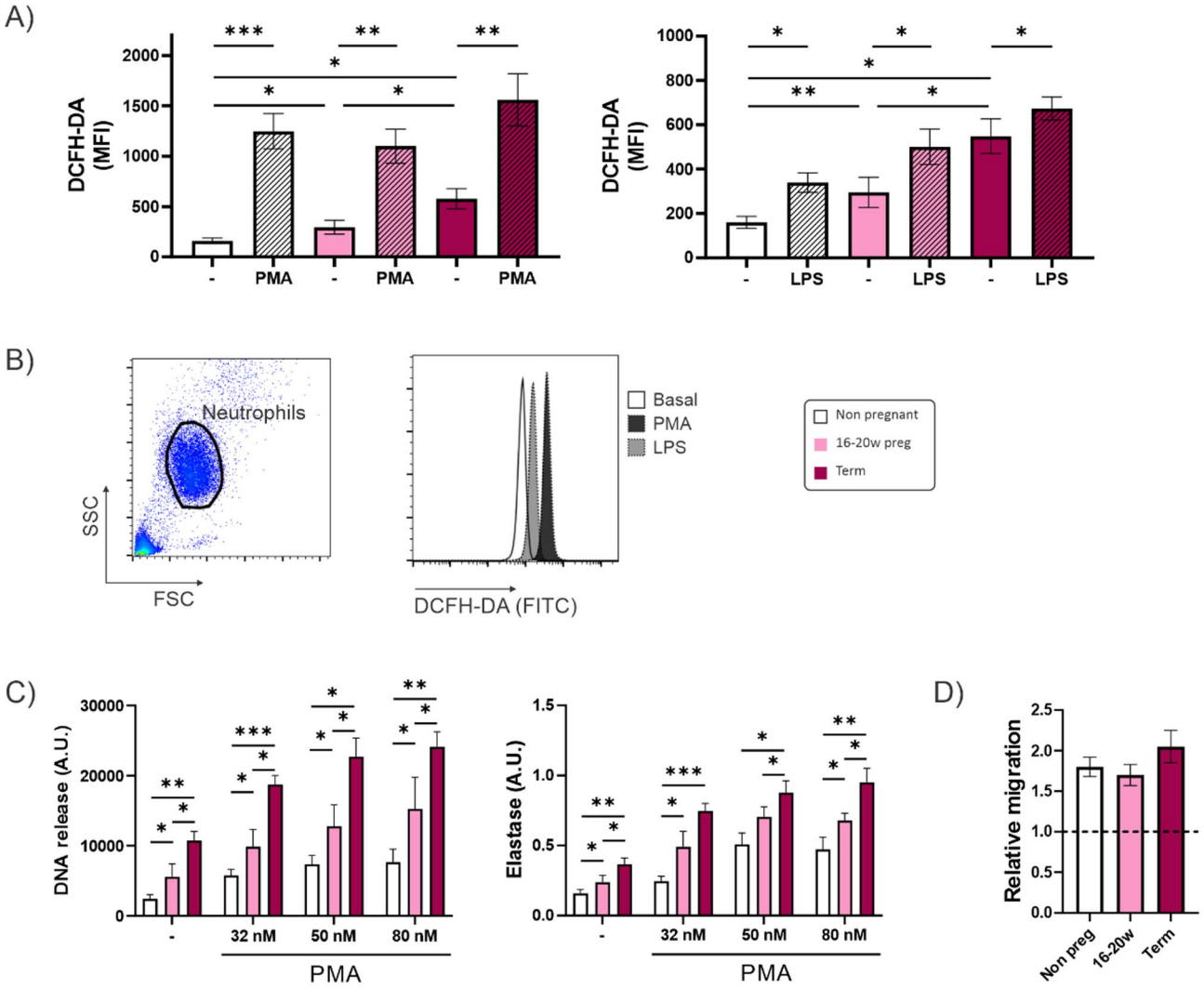

Fig. 2. ROS production and NETs release increase throughout pregnancy. Neutrophils from non-pregnant, 16–20-week pregnant, and term women were obtained and stimulated with PMA or LPS as shown for each case. (**A**) Neutrophils were stimulated for 45 min and then cultured with DCFH-DA fluorescent probe to assess ROS production by flow cytometry. (**B**) Representative dot plot of neutrophils after purification (left) and histogram of unstimulated and activated neutrophils (right). (**C**) Neutrophils were challenged with increasing PMA concentrations for 240 min and then DNA release and elastase activity were measured in their supernatants. (**D**) Neutrophils from different groups were placed on top of a 5 µm transwell chamber. After 60 min, cells that migrated were counted using a flow cytometer. (**A**–**D**) Each graph displays data from at least 8 samples per population. Values are mean ± SEM $*p<0.05$, $**p<0.01$, $***p<0.001$, Kruskal–Wallis followed by Dunn's multiple comparisons tests or Mixed-effects analysis were used.

correlated with bacteria content in the sample (Fig. 4E, left). There is also a positive correlation between the percentage of *P. gingivalis* and ROS production but not with the percentage of *F. nucleatum* (Fig. 4E), assessed with Spearman's correlation test. Regarding NET formation, both GCF from pregnant women at 16–20w and term, induced higher NET release, compared to GCF from non-pregnant women, shown as an increase in DNA release and elastase activity (Fig. 4F).

Discussion

Even though pregnancy complications associated with periodontitis have been widely documented, little is known about the extent and mechanisms by which gingival inflammation and pathogens contribute to the activation of circulating neutrophils and eventually to adverse pregnancy outcomes.

Successful gestation requires the continuous profiling of maternal neutrophils to ensure appropriate immune responses and maintain homeostasis. Most studies on neutrophils during pregnancy have primarily focused on the third trimester, with limited research exploring their profiles throughout the entire course of gestation[15,16]. This is partly due to their strong association with preeclampsia, a complication that typically appears after mid gestation[12]. For this reason and to better understand the transitional changes during pregnancy, we evaluated

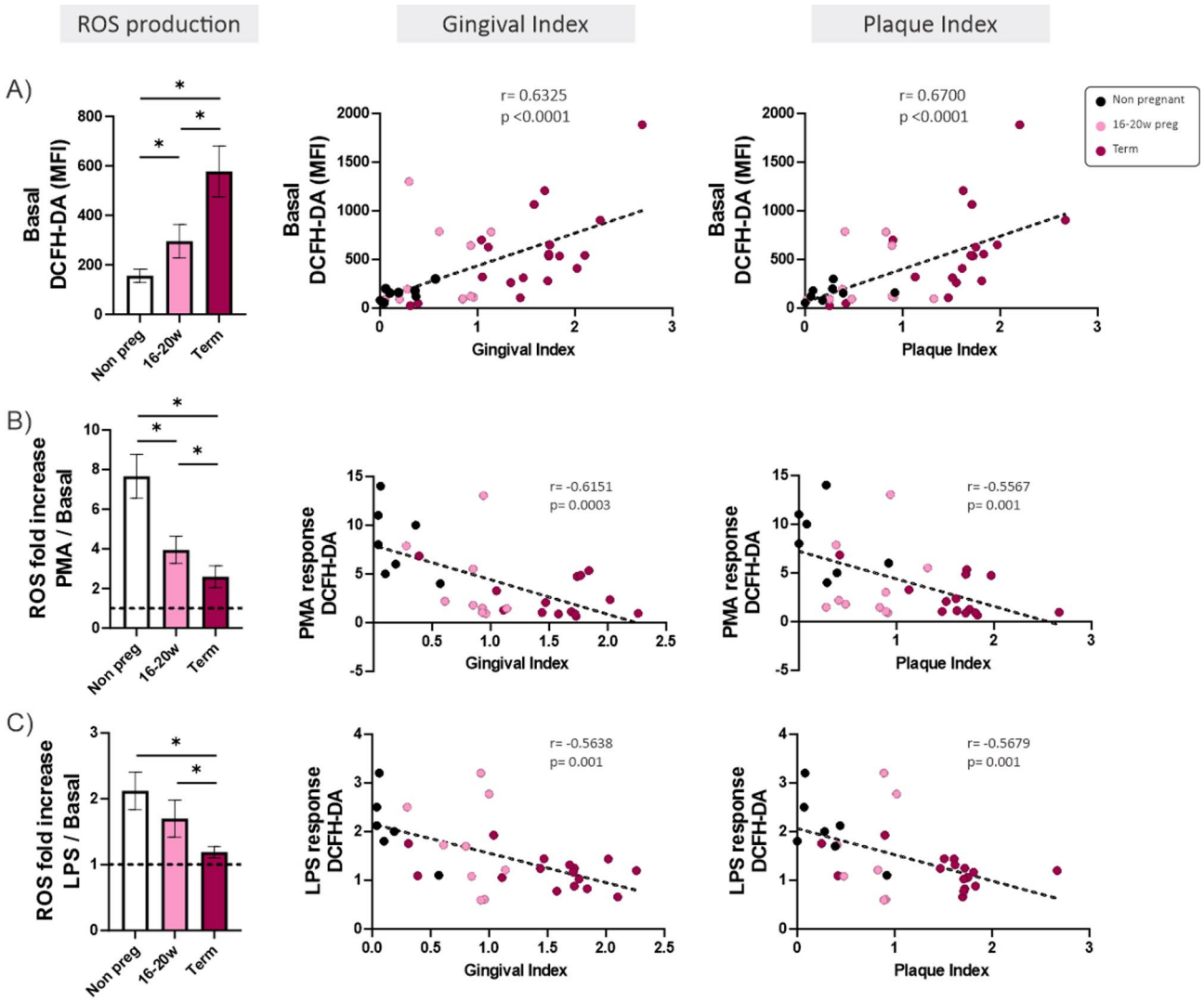

Fig. 3. ROS response through gestational age and gingival inflammation of the women. Neutrophils were isolated from each group, and they were left unstimulated (**A**) or activated with PMA (**B**) or LPS (**C**) as shown in each case. Neutrophils were stimulated for 45 min and then cultured with DCFH-DA to analyze ROS formation. Clinical features were assessed as indicated in materials and methods section. Subsequently, association analyses were performed between functional profile of neutrophils and clinical indexes using Spearman correlation. $p < 0.05$ in all cases. Panel (**A**) shows a positive correlation whereas (**B** and **C**) show negative correlation. Each dot represents a sample, with at least 7 samples per population in each graph. $^{*}p < 0.05$, Kruskal–Wallis followed by Dunn's multiple comparisons test was used for column graphics shown on the left.

non-pregnant women, women at 16–20 weeks of gestation, and those at term, along with a thorough assessment of their gingival inflammatory status.

Our results provide novel insight into the immunometabolic and functional modulation of neutrophils throughout pregnancy and their association with gingival inflammation of women. This conclusion is based on the following observations: First, pregnancy reprograms neutrophil metabolism, driving a progressive shift toward enhanced glucose utilization and increased lipid droplet accumulation toward the end of gestation. Second, unstimulated ROS production rises as gestation progresses and correlates with gingival inflammatory status; however, this production is partially restrained upon proinflammatory stimulation. Third, both basal and PMA-induced NET release increase with pregnancy. Finally, gingival crevicular fluid enhances neutrophil activation according to gestational stage. Fine temporal and spatial control of immune and metabolic pathways is crucial to meet the high-energy demands of pregnancy. The number of neutrophils increases throughout pregnancy, undergoing functional and metabolic changes. A proangiogenic regulatory decidual population has been described in the second trimester[23], while towards term, neutrophils acquire a more activated, pro-NETotic state[10,12,24,25]. In line with this, prior reports show elevated ROS production during pregnancy, which is further enhanced in obstetrical diseases such as maternal infection or preeclampsia[15]. Consistently across the patients evaluated here, neutrophils exhibited an activated profile characterized with increased NET release

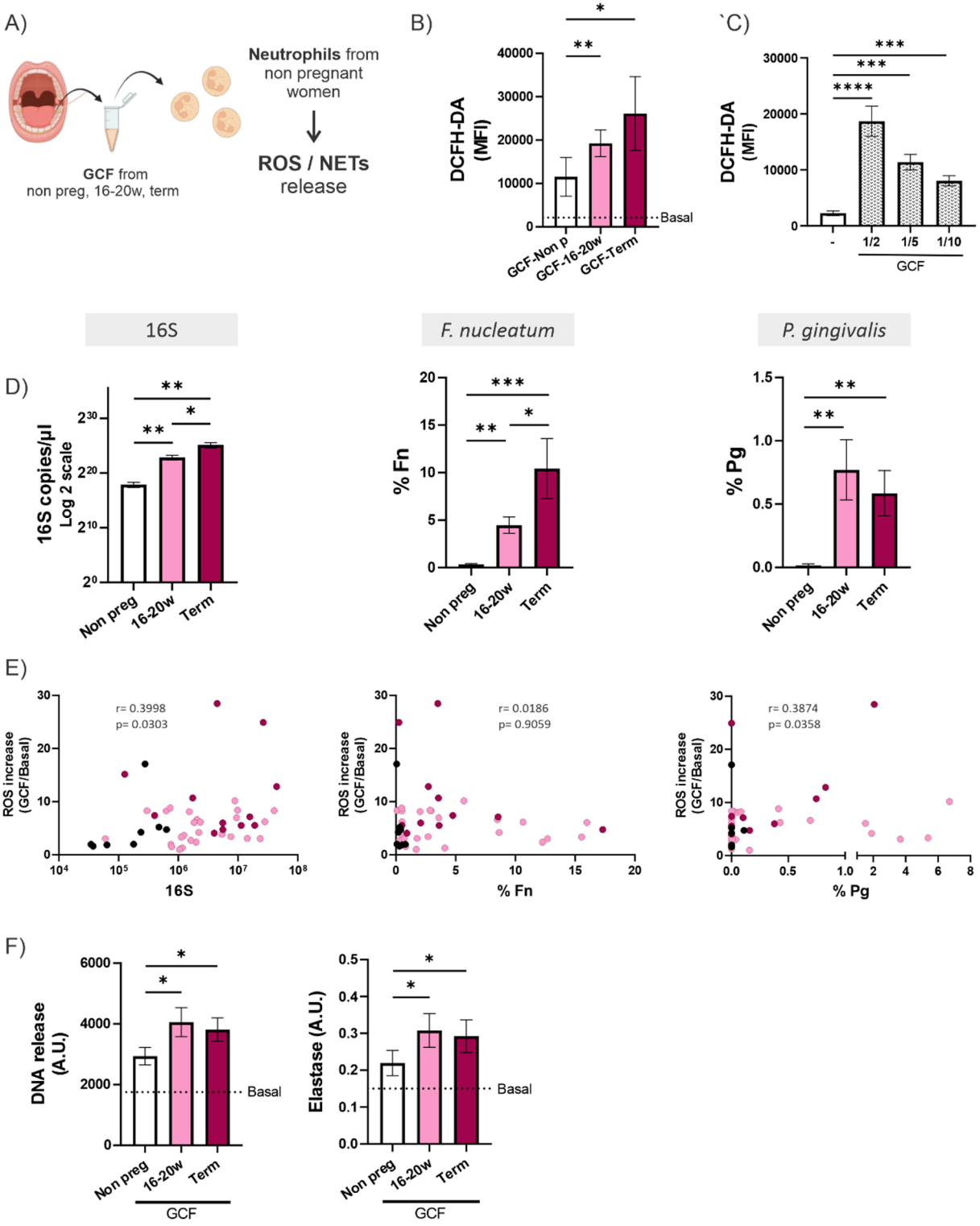

Fig. 4. Gingival crevicular fluid activates neutrophils. (**A**) Graphical scheme of the experiment set up. (**B**) Neutrophils from 10 non-pregnant control women were stimulated for 45 min with gingival crevicular fluid (GCF) from the three cohorts and ROS was assessed using DCFH-DA by flow cytometry. (**C**) Different GCF dilutions were used to stimulate neutrophils. (**D**) 16S copies/µl, *F. nucleatum* and *P. gingivalis* were determined in GCF from the three groups, by qPCR. (**E**) Association analyses were performed between ROS fold increase elicited by GCF and microbiological parameters using Spearman correlation. *p* and r values are shown for each graph. Each dot represents one GCF sample; at least 8 fluids of each population were tested. $p < 0.05$ for 16S and %Pg correlation analysis. For B, C, D and F, Kruskal–Wallis followed by Dunn's multiple comparisons test was used. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

and ROS production, which progressed throughout gestation and correlated with the worsening of the gingival status of the mother. Notably, neutrophils from women at term displayed the highest increase in glucose uptake upon stimulation, compared to those from 16 to 20 weeks of gestation and non-pregnant women, suggesting a metabolic shift that parallels their functional activation. The differential glucose uptake observed in parallel with the increase in ROS production at the different stages of pregnancy highlights the key role of metabolic reprogramming in finely tuning neutrophil function. The increase in glucose uptake would likely be diverted into the pentose phosphate pathway, providing reducing power (NADPH) to sustain ROS generation and redox balance.

In this sense, neutrophils tightly regulate glucose utilization and flux to sustain the immediate energy demands of a proportionate but effective host response[26,27]. Previous results from our lab indicate that trophoblast cells contributed to this modulation[17]. Conditioned media from trophoblast cells also inhibited PMA-induced NETosis partly by impairing glucose uptake in neutrophils.

Several studies have revealed that neutrophil function and NETs formation are highly, but not solely, dependent on glycolysis[28,29]. In line with these findings, our previous work showed that PMA-induced NETs were blocked by a fatty acid oxidation inhibitor in neutrophils from pregnant women, indicating a contribution of fatty acid metabolism to neutrophil activity during pregnancy[17]; based on this, we further assessed lipid metabolism. Upon activation, neutrophils from pregnant women were not able to increase the uptake of long chain fatty acids. However, neutrophils from women at term did increase lipid droplet accumulation after activation. Regarding stimulated neutrophils from 16 to 20 weeks pregnant women, they do not uptake the glucose analog at the same rate as neutrophils from women at term, nor accumulate lipid droplets, which supports the lesser level of ROS and NETs and accompanies the homeostatic, non-inflammatory role of neutrophils at this gestational stage. On the other hand, stimulated neutrophils from women at term are more activated and, in consequence, they need more fuel like glucose and lipids to meet their higher energy demands. Such a shift likely reflects the physiological need to prepare the maternal immune system for labor and to address the potential microbial challenges associated with delivery, highlighting the dynamic immunometabolic adaptations of neutrophils throughout gestation. To the best of our knowledge, this is the first publication to address the changes in neutrophil metabolic features (glucose and fatty acid uptake, and lipid droplet content) at different stages of pregnancy and their functional correlates. To determine whether pregnancy-related transient hormonal and metabolic changes, which in turn cause morphofunctional adaptations in the oral cavity, are associated with differential neutrophil profiles, we analyzed gingival inflammation and neutrophil parameters across cohorts.

Plaque and gingival indexes increase as gestation progresses, as well as bacteria load in the GCF, and they correlate with the aforementioned neutrophil activation. Gingival inflammation during pregnancy is a result from the combined effect of increased plaque accumulation and hormonal amplification of the inflammatory response. It is noteworthy that neutrophils from pregnant women exhibit a restrained ROS response compared to those from non-pregnant women. This modulation may help prevent an excessive inflammatory state, which would be detrimental to pregnancy and lead to adverse outcomes such as preterm birth, low birth weight, pre-eclampsia, and fetal growth restriction[2,30]. It has been shown that circulating neutrophils from patients with periodontitis are more activated but the mechanisms underlying this activation are elusive[31]. Since there is a persistent translocation of bacteria from periodontal tissues into circulation, extracellular vesicles and PAMPs release as well as damage signals from infected cells might be contributing to neutrophil peripheral shaping.

Numerous studies have confirmed that periodontal pathogens and their metabolites can contribute to adverse pregnancy outcomes through both direct and indirect mechanisms. Several species have been identified in the fetal–placental unit, including *A. actinomycetemcomitans*, *P. intermedia*, *C. rectus*, *T. forsythia*, *T. denticola*, and the most frequently detected: *P. gingivalis* and *F. nucleatum*[30]. Based on this evidence and given that these two species show the most consistent association with adverse obstetric outcomes—supported by large human cohorts and animal models linking systemic immune responses during pregnancy to such pathologies—we focused our study on *P. gingivalis* and *F. nucleatum*.

Porphyromonas gingivalis is a keystone pathogen in periodontal disease, with substantial evidence supporting its involvement in pregnancy-related complications[2,4,7]. This bacterium produces extracellular vesicles (outer membrane vesicles or OMVs), which are released into the circulation and can affect distant tissues and organs. This is one of the proposed mechanisms by which oral pathogens may contribute to neutrophil activation and dysregulation, as observed in our study. Using in vitro models with first-trimester human trophoblast cells and freshly isolated neutrophils, we have demonstrated that *P. gingivalis* OMVs promote neutrophil activation and migration[32]. Furthermore, in our in vitro model simulating the interaction between gingival components and circulating neutrophils during pregnancy, we demonstrated that GCF components promote NET and ROS release, both of which positively correlate with bacterial load and *P. gingivalis* abundance in the fluid. It is very interesting to note that, despite the increase in *F. nucleatum* throughout pregnancy, its abundance does not correlate with neutrophil activation. In line with this, neutrophils isolated from salivary samples induce NET formation and this response is highest during the third trimester[6]. Also, NET formation is reportedly higher in pregnant women with gingivitis, compared to non-pregnant or pregnant without oral pathology[6].

Evidence suggests that periodontal treatment may positively influence pregnancy outcomes, but well-designed randomized controlled trials are still required to confirm these findings. Our current findings provide novel insights into the immunometabolic, functional, and phenotypic changes of neutrophils throughout pregnancy. Future studies to identify and characterize specific bacterial mechanisms involved in neutrophil activation might certainly contribute to translational research in the field. Investigation on the metabolic and functional reprogramming of circulating neutrophils can help us understand the mechanisms that underlie successful pregnancies and might provide insights into developing diagnostic and therapeutic approaches for pregnancy-related diseases.

Limitations of the study

We typically obtained less than 5 mL of peripheral blood from term pregnant participants and sometimes from the 16 to 20w, limiting the number of experiments performed per sample so not all assays were possible on the same individual. Consequently, we were unable to perform immunofluorescence microscopy or measure citrullinated histone H3 (H3Cit), which is considered a more definitive marker of NET formation. Instead, we relied on measurements of extracellular DNA and neutrophil elastase activity, which are widely used surrogate markers of NET release, although not entirely specific.

To fully characterize neutrophils changes throughout pregnancy, longitudinal studies would be ideal. However, these are challenging, as many women change clinics or discontinue consultations before delivery. Including pathological cases would also be valuable to explore potential associations.

An additional limitation of this study is the absence of a control group of pregnant women without gingival inflammation. Given the high prevalence of pregnancy-associated gingivitis, particularly in our recruitment setting, nearly all pregnant participants exhibited some degree of gingival inflammation. Consequently, it was not feasible to isolate a subgroup of "healthy" pregnant women for comparison. Therefore, the neutrophil functional and metabolic changes observed should be interpreted as occurring in pregnancy in the presence of gingival inflammation.

Finally, the cross-sectional nature of this study precludes causal inference and does not capture longitudinal changes in the same individuals. Our results should therefore be interpreted as associations across pregnancy stages, rather than as direct evidence of temporal progression.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

C.P.L. and D.V. conceptualization and designed research. G.C., F.M., F.S., V.H., and B.L. performed metabolic, phenotypic, and functional experiments. L.R.D. and L.D. followed up with pregnant patients at their dental health control and obtained clinical history and samples from 16 to 20w pregnant women. They performed oral screening and collected GCF samples. L.G. performed microbiological analysis. S.N., A.S., L,C. and P.F. followed up term pregnant women and obtained clinical history and samples after cesarean section surgery. G.C. and F.M. analyzed data and statistics. A.S., R.R., D.V., and C.P.L., interpreted results of experiments. G.C. prepared figures and wrote the manuscript; D.V., and C.P.L. edited and revised the manuscript. All authors approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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