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Pregnancy associated epigenetic markers of inflammation predict depression and anxiety symptoms in response to discrimination

Femke Sluiter [a,1], Angela C. Incollingo Rodriguez [b,1], Benjamin C. Nephew [c], Ryan Cali [d], Chris Murgatroyd [a,*], Hudson P. Santos Jr. [e]

[a] Department of Life Sciences, Manchester Metropolitan University, Manchester, United Kingdom
[b] Department of Social Science & Policy Studies, Worcester Polytechnic Institute, Worcester, MA, United States
[c] Department of Biology and Biotechnology, Worcester Polytechnic Institute, Worcester, MA, United States
[d] University of Massachusetts Medical School, Worcester, MA, United States
[e] Biobehavioral Laboratory, School of Nursing, University of North Carolina at Chapel Hill, North Carolina, United States

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ABSTRACT

Latina mothers, who have one of the highest fertility rates among ethnic groups in the United States (US), often experience discrimination. Psychosocial influences during pregnancy, such as discrimination stress, promotes inflammation. However, the role of epigenetic markers of inflammation as a mediator between, and predictor of, maternal discrimination stress and neuropsychiatric outcomes has not been extensively studied. The current study investigates the role of DNA methylation at *FOXP3* Treg-cell-specific demethylated region (TSDR), as a marker of regulatory T (Treg) cells that are important negative regulators of inflammation, and the promoter of tumour necrosis factor-alpha (*TNF-α*) gene, an important pro-inflammatory cytokine, in relation to discrimination stress during pregnancy and depression and anxiety symptomatology.

A sample of 148 Latina women residing in the US (mean age 27.6 years) were assessed prenatally at 24–32 weeks' gestation and 4–6 weeks postnatally for perceived discrimination exposure (Everyday Discrimination Scale, EDS), emotional distress (depression, anxiety, perinatal-specific depression), acculturation, and acculturative stress. DNA methylation levels at the *FOXP3* and *TNFα* promoter regions from blood samples collected at the prenatal stage were assessed by bisulphite pyrosequencing.

Regression analyses showed that prenatal EDS associated with postnatal emotional distress, depression and anxiety symptoms only in those individuals with higher than mean levels of *FOXP3* TSDR and *TNFα* promoter methylation; no such significant associations were found in those with lower than mean levels of methylation for either. We further found that these relationships were mediated by *TNFα* only in those with high *FOXP3* TSDR methylation, implying that immunosuppression via *TNFα* promoter methylation buffers the impact of discrimination stress on postpartum symptomatology. These results indicate that epigenetic markers of immunosuppression and inflammation play an important role in resilience or sensitivity, respectively, to prenatal stress.

1. Introduction

Latina immigrants in the United States often experience high levels of psychosocial stress during pregnancy (Jill Fleuriet and Sunil, 2014). Studies indicate that such psychosocial stress is linked to impaired physical and mental health, particularly increased levels of psychiatric symptoms related to depression and anxiety (Harris and Santos, 2020; Lara-Cinisomo et al., 2016; Perry et al., 2013). Considering that Latina women have among the highest rates for both birth and immigration of any minority group in the United States (Martin et al., 2019), it is important to understand the mechanisms linking stress and perinatal health in this group.

We have previously reported that discrimination exposure during prenatal and postnatal periods in Latina mothers correlates with DNA methylation of stress-related genes, including *NR3C1*, *BDNF* and *FKBP5* (Santos et al., 2018). It is also important, though, to evaluate immune pathways when considering that inflammation is strongly implicated in the etiology and pathophysiology of depression and stress (Maldonado

* Corresponding author. Bioscience Research Centre, Manchester Metropolitan University, Chester Street, Manchester, M1 5GD, United Kingdom.
E-mail addresses: c.murgatroyd@mmu.ac.uk (C. Murgatroyd), hsantos@unc.edu (H.P. Santos).
[1] These authors contributed equally.

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et al., 2018; Miller and Raison, 2016; Quinn et al., 2020). Moreover, relevant to this maternal population, inflammation is differentially regulated during pregnancy (Bränn et al., 2019) and also varies among ethnic groups, acculturation and socioeconomic status (Muscatell et al., 2018; Schmeer and Tarrence, 2018).

Immunological changes occur naturally during pregnancy, with alterations in immune cells and plasma cytokines (e.g., Bränn et al., 2019). Along with natural fluctuations, there is mounting evidence that psychological stress can activate inflammatory responses as well (Maydych, 2019). Namely, different types of stressors can elicit increases in inflammatory activity through release of proinflammatory cytokines that may induce depressive symptoms (Slavich, 2014). Stress from everyday discrimination, in particular, seems to engage inflammatory responses, especially in women. For instance, one study found that among Black and White women, those who reported experiencing discrimination had higher levels of c-reactive protein (CRP) than those who did not (Cunningham et al., 2012). In another study, higher levels of discrimination were associated with higher levels of interleukin-6 (IL-6) among women (Kershaw et al., 2016), and acculturation has been linked to increased IL-6 concentrations across pregnancy (Scholaske et al., 2018). Exposure of pregnant African American women to racial discrimination is associated with elevated levels of IL-4 and IL-6, suggesting allostatic overload which results in physiological fatigue (Giurgescu et al., 2016), and increased IL-6 has been documented in both pregnant and non-pregnant African American women in response to acute social stress (Christian et al., 2013). There is evidence that these race-related patterns in immune states and responses may be mediated by differential effects of sleep disturbances (Carroll et al., 2020). However, it should be noted that stress can induce both increases and decreases in immune factors (including immunosuppression), and/or a dysregulation in critical interactions between the hypothalamic-pituitary adrenal (HPA) and immune axes (Herbert and Cohen, 1993; Nakata, 2012; O'Connor et al., 2020; Yamakawa et al., 2009). Altered inflammatory relationships may explain the well-established link between discrimination and depression among Latinx individuals (Flores et al., 2008; Torres, 2009; Torres and Ong, 2010) including in the context of pregnancy (Incollingo Rodriguez et al., 2019), as there is evidence that certain types of acculturative stress are related to depressive symptoms in Latina mothers (Hill et al., 2019).

Two important markers of the immune response to perinatal discrimination and stress are tumour necrosis factor-alpha (TNF-α) and Forkhead Box P3 (FOXP3). TNF-α is a proinflammatory cytokine regulated through methylation at the promoter region (Sullivan et al., 2007) in macrophages/monocytes during acute inflammation (Leija-Martínez et al., 2020). In laboratory-based manipulations, acute stress promotes the release of proinflammatory cytokines such as TNF-α (Marsland et al., 2017; O'Donnell et al., 2008). Additionally, the cytokine response to social stress has been implicated in depression etiology in female adolescents (Giletta et al., 2018; Slavich et al., 2020). It has been suggested that TNF-α mediates adverse inflammatory-associated health effects of racial/ethnic discrimination (Carlson et al., 2017). For instance, a study of maternal depression reported that cerebrospinal fluid and blood levels of TNF-α were positively associated with postnatal-depressive mood (Boufidou et al., 2009). Other investigations, however, did not observe significant associations between TNF-α and prenatal depression and/or stress (Christian et al., 2009; Karlsson et al., 2017). Studies have also shown that the *TNF-α* gene can be epigenetically regulated by DNA methylation (Gowers et al., 2011) particularly at the region covering the promoter both during development and in response to stimulation that actively regulates its expression (Sullivan et al., 2007). Epigenetic regulation at the *TNF-α* promoter is also cell-specific, occurring in macrophages/monocytes during acute inflammation (Leija-Martínez et al., 2020).

Within intron 1 of the *FOXP3* gene, a Treg-cell-specific demethylated region (TSDR) in its unmethylated form confers constitutive expression of *FOXP3* in naive T cells, ensuring that they develop into regulatory T (Treg) cells (Floess et al., 2007). *FOXP3* TSDR demethylation is needed for Treg cell differentiation and represents a negative regulator of immune response (Bending and Ono, 2019). Studies demonstrate that, during inflammation, Tregs can lose their phenotypic properties and convert into effector T cells secondarily to the loss of *FOXP3* expression, *for review see* (Sawant and Vignali, 2014). The main role of Tregs is to reduce inflammation through the secretion of anti-inflammatory cytokines. Both human and animal studies show an association between an increased risk of major depression and a decreased number of Tregs (for review see Ellul et al., 2018). Increases in Tregs have also been linked to effective antidepressant treatment, suggesting they may be a mechanism of successful treatment (Grosse et al., 2016a; Himmerich et al., 2010). In a rodent model of maternal depression, rats exhibit reduced Tregs together with increases in postnatal proinflammatory cytokines, and fluoxetine appeared to attenuate this dysfunction (Li et al., 2016). Several human studies have found a decrease in Tregs in depressed patients (Chen et al., 2011; Grosse et al., 2016b; Li et al., 2010) while acute psychological stress has been shown to cause a reduction in Treg cells (Sommershof et al., 2009). A similar study reported increased *FOXP3* TSDR methylation, suggestive of reduced Treg cells, in female patients, but not males, with panic disorder (Prelog et al., 2016). Specific to the pregnancy context, a human study observed that Treg levels were significantly increased in prenatal and postnatal mothers who had postnatal depressive symptoms (Krause et al., 2014). TNF-α is able to increase expansion, stability, and possibly function of Tregs *via* the TNF receptor type 2 (Salomon et al., 2018).

Despite this strong theoretical basis to suggest these factors are related, few studies have investigated the associations between discrimination stress in Latina mothers and the regulation of immune markers, and there have been no investigations, to our knowledge, of epigenetic marking of immune-regulatory genes in mothers in the context of discrimination and acculturative stress and/or maternal mental health. The present study investigated DNA methylation of *FOXP3* TSDR and *TNF-α* promoter and measures of discrimination, acculturation, and emotional distress in a population of migrant Latina mothers in the US. It was hypothesized that epigenetic regulation of *FOXP3* and *TNF-α* as markers of inflammation during pregnancy may regulate and serve as indicators for adverse depression and anxiety symptoms in response to discrimination-related stress.

2. Material and methods

2.1. Participants

Healthy pregnant Latinas (n = 150) living in North Carolina were enrolled in the study between May 2016 to March 2017. Eligibility criteria included: (1) 18–45 years old, (2) Spanish- or English-speaking, (3) carrying a singleton pregnancy, (4) available for follow-up at 6 weeks postpartum. Exclusion criteria were: (1) currently experiencing severe depressive symptoms as determined by psychiatric interview, (2) history of psychotic or bipolar disorder, or receiving psychiatric pharmacotherapy or psychotherapy, (3) substance dependence in the last two years, (4) current foetal anomaly, or (5) current life-threatening conditions. These exclusions were adopted to avoid confounders and control for severe mood symptoms with onset before the study timeframe. Full details on recruitment procedures have been previously described (Santos et al., 2018). In summary, women were recruited during their prenatal clinical appointment visits by a trained bilingual staff in North Carolina, US. Data collection was completed in English or Spanish, depending on participants preference at the prenatal visit at 24–32-week gestation and at postnatally at 4–6 weeks postpartum. Measures used in this study had validated versions in English and Spanish. The Institutional Review Board of the University of North Carolina at Chapel Hill approved this study (#15–3027).

2.2. Measures

Everyday Discrimination Scale (EDS): a nine-item questionnaire was used to measure routine, day-to-day experiences of discrimination at the prenatal and postnatal time points, as previously described (Santos et al., 2018). This is a widely used measure of subjective experiences of discrimination (Williams et al., 1997), with a validated Spanish translation (Campo-Arias and Herazo, 2015). It correlates with measures of institutional racial discrimination and interpersonal prejudice (Krieger et al., 2005) and does not prime the subjects to think about race, which limits cues to prejudice prior to responding to the questions (Deitch et al., 2003). The 9-item Likert response scale for frequencies ranged from 0 ("never") to 5 ("almost every day"). An additional question asks the respondent to select a reason to which they attribute their experiences of discrimination (e.g., skin color, ethnicity). As previously described (Santos et al., 2018), we constructed a mean summary that ranged from 0 to 5, with a higher score indicating a higher frequency of perceived discrimination. Cronbach's alpha for item consistency for the EDS in our sample was 0.86 for T1 and 0.89 for T2.

Acculturative Stress Scale (ACS): The 9-item ACS was used to assess subjects' experience with the acculturation process, how well one adapts to the changes that are occurring, some of which are not under one's control (Gil, 1994). Respondents answer the frequency of emotions and experiences regarding acculturation to the US in the past year on a 1–5 Likert scale (1 = Not at All to 5 = Almost Always). Sample questions include "*How often do you feel that you would rather be more American if you had a choice?*" and "*How often do you feel uncomfortable having to choose between non-Hispanic/Latino and Hispanic/Latino ways of doing things?*". The sum of responses was averaged resulting in a score from 1 to 5, with higher score indicating a greater of acculturative stress.

Bidimensional Acculturation Scale (BAS): Acculturation was measured using the BAS to assess the degree to which individuals participate in the cultural domains of both the original and the culture of contact (Marin and Gamba, 1997). The BAS includes 24 questions to assess acculturation within both Hispanic (12 questions) and Non-Hispanic (12 questions) cultural domains and includes three subscales language use (6 questions), language proficiency (12 questions), and electronic media (6 questions). The BAS asks participants to report the frequency in which they experience events or their ability to use technology with 1–4 Likert scale, with higher scores indicating higher frequency or better ability (1 = Almost Never to 4 = Very Well). Participant responses from each cultural domain are summed and averaged resulting in a Hispanic BAS score as well as a Non-Hispanic BAS score between 1 and 4, with an overall score of 4 indicating a higher degree of acculturation. The BAS has been validated in both English and Spanish (Marin and Gamba, 1997).

Depressive and negative mood symptoms: The Inventory of Depression and Anxiety Symptoms (IDAS), a 99-item questionnaire (Watson et al., 2012), was used to measure depressive and negative mood symptoms at prenatal and postnatal time-points. Higher IDAS scores indicate more severe symptoms. Typical scores are 32.4 and 37.4 for control and high-risk women, respectively, and between 44.6 and 57.3 for depressed women (Segre et al., 2015). Cronbach's alpha for item consistency for the IDAS in our sample was >0.78 for prenatal and postnatal time points. The Generalised Anxiety Disorder Assessment (GAD-7), a seven-item questionnaire, was used to assess the severity of generalised anxiety disorder (Spitzer et al., 2006). Each item asks the individual to rate the severity of his or her symptoms over the past two weeks. Response options include "not at all", "several days", "more than half the days" and "nearly every day". The Edinburgh Postnatal Depression Scale (EDPS) was used to identify women who may have perinatal and/or postpartum depression symptoms (Cox et al., 1987). Each answer is given a score of 0–3 with a maximum score of 30.

2.3. DNA methylation

To minimize variability in stress, the study blood draw was incorporated into the routine prenatal blood draw at T1 followed by self-report measures. A 6 ml blood sample was drawn from a peripheral vein into a chilled EDTA-vacutainer, placed immediately on ice, and processed. The buffy coat was separated by centrifugation, frozen on dry ice, and stored at−80 °C at the University of North Carolina Biobehavioral lab. DNA extraction was performed with the QIAamp DNA Blood Mini Kit and extracted DNA was stored at−80 °C in individual cryovials until shipment. The extracted DNA was transported on dry ice to Manchester Metropolitan University for DNA methylation analysis. DNA methylation levels were determined by bisulphite pyrosequencing (PCR). Briefly, 1 μg DNA were treated using the EpiTect Bisulfite Kit (Qiagen) and amplified using the PyroMark PCR Kit using primers against candidate specific CpGs. The *TNF-α* promoter (Gowers et al., 2011) was analysed using primers (Forward, 5′-Biotin-GGGGTA TTTTTGATGTTTGTGT-3'; Reverse, 5′-CCTTAATAAAAAAACCCATAAA CTCAT-3′) with the PCR conditions 92 °C: 30 s, 62 °C: 30 s, 72 °C: 30 s, and sequenced with the primer 5′-AAACCCATAAACTCATCTA-3′ used to analyse the sequence 5′-AAAAAAACRA TAATAAACCC TACACCTTCT ATCTCRATTT CTTCTCCATC RCRAAAACRA AAATTTAAAA AAT-TAAAAAC ACA-3'. The *FOXP3* TSDR (Floess et al., 2007) was analysed using with primers (Forward, 5′-TTATTTGGGTTAAGTTTGTTG-TAGG-3'; Reverse, 5′- Biotin-CTACATCTAAACCCTATTATCACAAC-3′) with the PCR conditions 92 °C: 30 s, 62 °C: 30 s, 72 °C: 30 s, and sequenced using the primer 5′-GTGGTGTAGATGAAGT-3′ to analyse the sequence 5′- CCGGCGCATC CGGCCGCCAT GACGTCAATG GCGGA AAAAT CTGGGCAAGT CGGGGG-3'. See Supplementary Fig. 1 for location of the regions sequenced within *FOXP3* TSDR and promoter of *TNF-α*. We focused only on specific CpGs supported by previous literature to maintain statistical power and reduce effects of multiple analyses. Single-stranded biotinylated product was purified by mixing 10 μl of the amplification mixture, 2 μl of streptavidinsepharose HP (Amersham Biosciences), and 40 μl of binding buffer. The sepharose beads containing the immobilized biotinylated product were purified, washed, and denatured in 0.2 mol/l NaOH and washed again using the Pyrosequencing Vacuum Prep Tool (Qiagen). The biotinylated DNA was resuspended in 12 μl of annealing buffer containing 0.3 μmol/l pyrosequencing primer (*see above*) and quantified by pyrosequencing using the PSQ 24 MA system with the PyroMark Q24 Advanced CpG Reagents (Qiagen). The percentage methylation for each of the CpG sites was calculated using Pyro Q-CpG software (Qiagen). All analyses represent the average of three separate assays from PCRs performed in triplicate and sequenced.

Regarding the effects of blood cell composition on DNA methylation, *FOXP3* TSDR demethylation is found to be restricted to Treg cells when tested against all major peripheral blood cell types and a selection of non–blood cells (Floess et al., 2007). Examining average methylation of the specific CpGs in the TNF-α promoter (Supplemental Fig. 2) in $CD4^+$ T cells, monocytes, and neutrophils from over 100 control subjects cell types from human whole blood, using the dimethyl Database (http://imethyl.iwate-megabank.org/index.html) (Hachiya et al., 2017) we find average methylation levels are relatively similar of 9.7%, 12.5% and 8.6%, respectively. *FOXP3*, is located on the X chromosome, though in this study all subjects were female so we did not have to control for sex-differences.

2.4. Statistical analyses

A series of linear regression analyses were performed to test associations among DNA methylation markers, maternal acculturation and acculturative stress, and maternal depressive and negative mood

symptoms. The following variables were examined as potential covariates by testing for relationships with the above variables of interest: maternal age, marital status, education level, and income. Of these, the only significant association was between maternal age and acculturative stress. Therefore, all tests including acculturative stress controlled for maternal age. All other associations between variables and covariates were null (*p*-values ranging from 0.11 to 0.97).

To determine the moderating effect of DNA methylation patterns, relationships between maternal acculturation and acculturative stress and mood symptomatology were also examined in stratified models by high versus low methylation status. Here, the cut-offs for high FOXP3 and *TNF-α* methylation were 88.78 and 20.01, respectively.

Finally, given the interrelatedness of the above constructs and the longitudinal nature of these data, moderated mediation analyses were conducted to represent an integrated model. First, hierarchical linear regression analyses tested TNF-α methylation as a mediator of the relationship between prenatal discrimination and the following postnatal outcomes: general depressive symptoms, perinatal-specific depressive symptoms, and generalised anxiety symptoms. These models were run for the overall sample. Second, considering the significant negative association between *FOXP3* and *TNF-α* methylation, the sample was stratified by *FOXP3* methylation status (higher versus lower than the mean value of 88.77), and these mediation analyses were tested separately in each group. None of the above covariates were significantly related to any constructs in these models, and therefore were excluded from these analyses.

Statistical significance was determined at alpha 0.05. To account for alpha accumulation across multiple tests conducted in this analytic plan, a false discovery rate analysis (Benjamini and Hochberg, 1995) was conducted for each set of tests.

All analyses were completed in SPSS V26 software.

3. Results

3.1. Characteristics of the participants

At the time of assessment, the mean age of the cohort was 27.7 (SD 6.4) years, and the majority (125 of the 148) were not born in the US. Most of the participants were either living together with a partner or married (74.4%) (Supplementary Table 1). Concerning discrimination, 44.2% of the women reported that they had experienced discrimination and related their discrimination experiences mostly to their race and ancestry (Santos et al., 2018). See Supplementary Table 2 for scores for the EDS questionnaire.

3.2. Association between FOXP3 TSDR and TNF-α promoter methylation

Linear regression analysis revealed a significant negative association between levels of *FOXP3* and *TNF-α* methylation levels (B −0.21, β −0.170, $p = 0.039$). These markers suggest that increased Treg cells associate with decreased *TNF-α* expression.

Table 1
Regression analyses predicting *FOXP3* TSDR and *TNF-α* promoter DNA methylation based on discrimination and acculturation.

FOXP3 methylation	B	SE B	β	*p*-value
Prenatal EDS	-.187	.133	-.116	.161
Total Acculturation	−0.46	.58	-.80	.427
Hispanic Domain	0.79	.53	.12	.134
Non-Hispanic Domain	−0.42	.27	-.13	.130
Acculturative Stress* (Prenatal)	−0.004	.04	-.01	.956
***TNF-α* methylation**				
Prenatal EDS	0.11	.06	.15	.076
Total Acculturation	1.67	.70	.19	.019
Hispanic Domain	0.45	.65	.06	.685
Non-Hispanic Domain	0.63	.34	.15	.062
Acculturative Stress* (Prenatal)	−0.12	.08	-.12	.162

*Note. These analyses controlled for maternal age.

3.3. FOXP3 TSDR and TNF-α promoter methylation association with discrimination, acculturation, and acculturation stress

Regression analyses revealed no significant associations between methylation of the *FOXP3* gene and prenatal everyday discrimination, $r(148) = -0.12$, $p = 0.161$. There were also no significant associations between levels of acculturation and acculturative stress and *FOXP3* methylation . Total acculturation, however, was a significant positive predictor of *TNF-α* methylation (Table 1). Prenatal discrimination was marginally associated with *TNF-α* methylation, such that discrimination levels predicted higher methylation. Acculturative stress was not predictive of *TNF-α* at either pre- or postnatal time points (Table 1).

3.4. Depression and TNF-α TSDR and FOXP3 promoter methylation

Regression analyses revealed that prenatal IDAS was negatively associated with *FOXP3* methylation levels ($r(148) = -0.180$, $p = 0.28$). However, there were no other significant associations with any of the other depression and anxiety measures (Table 2). When testing for *TNF-α,* postnatal EPDS positively associated with methylation levels (Table 2).

3.5. Influence of TNF-α TSDR and FOXP3 promoter methylation on the association between stress (ACS and EDS) and anxiety and depression

We hypothesized that an epigenetic inflammatory profile might influence stress responsivity, in particular how an individual might respond to a stressor with increased levels of depression and anxiety. Individuals were grouped based on having higher or lower than the mean*TNF-α* and *FOXP3* methylation levels, for each variable respectively. We then tested for differences in stress, depression, and anxiety based on methylation level group (high versus low). This revealed that prenatal EDS associated with postnatal depression and anxiety across multiple measures (EPDS, IDAS, GAD) only in those individuals with higher *TNF-α* methylation. Postnatal EDS also significantly positively associated with postnatal GAD, EPDS and IDAS only in those individuals with the higher *FOXP3* methylation (Table 3). This suggests that in those individuals with lower *TNF-α* expression and lower Tregs, higher levels of perceived stress, in the form of discrimination and acculturation, is associated with increased anxiety and depressive symptoms postnatally.

3.6. Mediation and moderation of TNF-α TSDR and FOXP3 promoter in stress to postnatal symptom relationships

Since we observed that *TNF-α and FOXP3* methylation during

Table 2
Regression analyses of *FOXP3* TSDR and *TNF-α* promoter DNA methylation with depression and anxiety.

FOXP3 methylation	B	SE B	β	*p*-value
Prenatal GAD	−0.007	.088	-.006	.939
Prenatal IDAS	−0.021	.009	-.180	.028
Prenatal EPDS	−0.085	.077	-.092	.268
Postnatal GAD	0.056	.092	.052	.544
Postnatal IDAS	-.008	.010	.066	.443
Postnatal EPDS	0.123	.074	.141	.098
***TNF-α* methylation**				
Prenatal GAD	−0.16	.108	.12	.884
Prenatal IDAS	0.14	.012	.096	.248
Prenatal EPDS	−0.67	.095	.059	.479
Postnatal GAD	0.156	.114	.117	.172
Postnatal IDAS	.020	.013	.138	.104
Postnatal EPDS	0.189	.091	.173	.041

Table 3
Regression analyses of prenatal discrimination (EDS) as a continuous predictor of postnatal symptoms stratified by higher or lower than average *TNF-α* promoter or *FOXP3* TSDR methylation status.

TNF-α methylation					
	Outcome	*B*	*SE B*	*β*	*p*-value
HIGH methylation	preEDS postGAD	.531	.219	.324	.019
	preEDS postIDAS	.103	.026	.489	<.0001
	preEDS postEPDS	.434	.171	.338	.014
LOW methylation	preEDS postGAD	.200	.199	.108	.317
	preEDS postIDAS	.024	.018	.145	.181
	preEDS postEPDS	.099	.175	.061	.573
FOXP3					
	Outcome	*B*	*SE B*	*β*	*p*-value
HIGH methylation	preEDS postGAD	.489	.114	.440	<.0001
	preEDS postIDAS	.091	.015	.568	<.0001
	preEDS postEPDS	.437	.087	.502	<.0001
LOW methylation	preEDS postGAD	.404	.340	.153	.240
	preEDS postIDAS	.037	.026	.180	.166
	preEDS postEPDS	.322	.316	.131	.313

pregnancy predicted postpartum symptomatology following prenatal stress, we tested for mediation effects overall and in those individuals with high or low *FOXP3* methylation. See Fig. 1 for regression coefficients for the models summarized below. In the overall sample, *TNF-α* methylation did not significantly mediate the relationship between prenatal EDS and postnatal IDAS. However, among participants above the mean in *FOXP3* methylation, prenatal *TNF-α* methylation did significantly mediate the relationship between prenatal EDS and postnatal IDAS. After accounting for the effect of the mediator, prenatal EDS still uniquely predicted postnatal IDAS, indicating partial mediation. No paths were significant among participants with *FOXP3* methylation below the mean. A similar pattern was seen for prenatal EDS and postnatal EPDS.

TNF-α methylation did not significantly mediate the relationship between prenatal EDS and postnatal GAD. However, among participants above the mean on *FOXP3* methylation, *TNF-α* methylation did significantly mediate the relationship between prenatal EDS and postnatal GAD. Similar to the above, this model suggests partial mediation. No paths were significant in participants with *FOXP3* below the mean.

Therefore, we found that for people with high *FOXP3* methylation (i.e. a marker of lower TReg cells), prenatal EDS significantly predicts EPDS/IDAS partially via a pathway through TNF-*α*. That this model is not significant with low *FOXP3* methylation supports that in this group, discrimination stress does not correlate with increased risk of postpartum symptomatology.

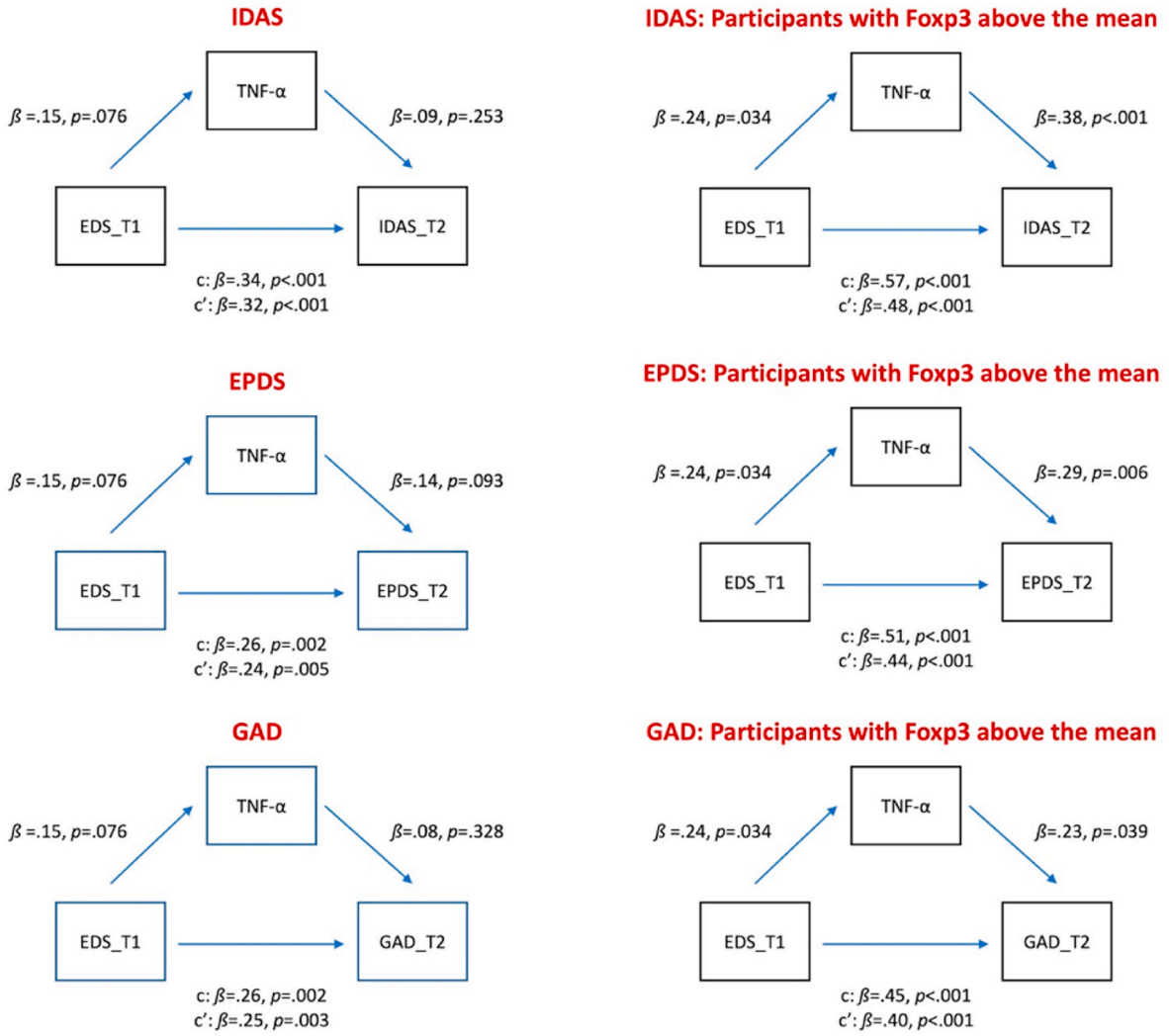

Fig. 1. Hierarchical linear regression analyses testing *TNF-α* methylation as a mediator of the relationship between prenatal discrimination and postnatal general depressive and generalised anxiety symptoms stratified by high and low *FOXP3* methylation levels. IDAS, Inventory of Depression and Anxiety Symptoms; GAD-7, Generalised Anxiety Disorder Assessment; EPDS, The Edinburgh Postnatal Depression Scale.

3.7. False discovery rate analyses

The previously significant tests reported in Tables 1 and 2 neared but did not surpass the corrected thresholds. All results reported in Table 3 and the moderated mediation pathways tested in Fig. 1 did surpass the corrected threshold with the exception of *TNF-α* mediating the path from prenatal EDS to postnatal GAD among people high in high *FOXP3*. However, this test also neared the corrected threshold. See Supplementary Table 3 for full results of the false discovery rate analyses.

4. Discussion

The present study examined whether epigenetic marking of immune-related genes mediate adverse depression and anxiety related symptomotology following discrimination stress exposure during pregnancy. Results revealed that prenatal exposure to discrimination was associated with adverse outcomes for postnatal mental health only in those individuals with higher than mean levels of *TNF-α* promoter and *FOXP3* TSDR methylation. No such significant associations are found in those with lower than mean levels of either *TNF-α* and *FOXP3* methylation. The predictive relationship between prenatal discrimination and postnatal depression, anxiety, and emotional distress was also mediated by *TNF-α* methylation in those with high *FOXP3* methylation. These results suggest that epigenetic markers of increased levels of Treg cells and decreased TNF-α levels - indicative of immunosuppression - mediate susceptibility and resilience to the effects of discrimination and acculturation stress, postnatal depression and anxiety etiology (Supplementary Fig. 2).

Altered immune activity, whether accentuated or suppressed, is a critical mechanism in a host of pathologies, altering how the body adapts to its environment and associated stimuli. In the present study, the *TNF-α* and *FOXP3* data suggest that higher TReg cells, suggestive of reduced inflammation, inhibits the association between prenatal stress and postnatal depression and anxiety symptoms. These findings are consistent with numerous rodent and human studies suggesting that Tregs may play a protective role against depressive-like behaviour (for review see Ellul et al., 2018). In a rat model of postnatal depression, Tregs cell counts were reduced; this was negatively associated with pro-inflammatory cytokines, and antidepressant treatment increased the levels of Tregs (Li et al., 2016). Several human studies have also found a decrease in Tregs in patients with major depression (Chen et al., 2011; Grosse et al., 2016b; Li et al., 2010) while acute psychological stress has been shown to cause a reduction in Treg cells (Sommershof et al., 2009). Furthermore, numerous studies and meta-analyses support the hypothesis of increased *TNF-α* in depression (Dowlati et al., 2010). In maternal depression, one study found that cc ccerebrospinal fluid and blood levels of TNF-α positively associated with postnatal-depressive mood (Boufidou et al., 2009) while another study observed a non-significant trend for higher levels of TNF-α with prenatal depression (Christian et al., 2009). In contrast, the present data indicate that decreased levels of *TNF-α* expression in the prepartum period are associated with postpartum depression and anxiety symptomotology in the high *FOXP3* levels of methylation subgroup. Taken together with our previous published findings on increased stress related gene expression during the prepartum period, it is hypothesized that increased levels of discrimination stress stimulate increased HPA activity and subsequent immune suppression, resulting in increased *TNF-α* methylation and decreased expression.

Another aspect to this study suggests that an altered inflammatory profile can modulate the risk of developing depression and anxiety symptoms following stress. This supports the hypothesis that resilient individuals have a different immunophenotype from that of stress susceptible individuals (Dantzer et al., 2018). It is possible to make susceptible individuals resilient and vice versa by changing their inflammatory phenotype. Numerous studies indicate that patients with inflammatory diseases have increased risks of developing depression and stress-related disorders, *for review see* (Leonard, 2010). Also, patients treated with cytokine therapies are at increased risk of developing depression (e.g., Raison et al., 2005). Conversely, a recent systematic review suggests that anti-inflammatory agents play an antidepressant role in patients with major depressive disorder (Bai et al., 2020). A rodent model of depression-like behavior following chronic unpredictable mild stress reported increased Treg cells (Hong et al., 2013). Furthermore, a more recent mouse study, testing for resilience and susceptibility to chronic stress using a model of social defeat, observed that social stress suppressed Treg cell differentiation to a greater degree in those mice more susceptible to the stress (Ambrée et al., 2019). We therefore hypothesize that individuals with lower Treg levels in the current study are more susceptible to chronic discrimination stress during the prenatal period, and those with lower *FOXP3* methylation and high Treg levels more resilient.

A final consideration is the potential effect of social support on ethnic minorities suffering from stress and depression and anxiety symptoms. Social support is known have a role in the effects of discrimination on mental health (Ajrouch et al., 2010; Prelow et al., 2006) especially in Latinx populations (Finch and Vega, 2003). Social isolation induces robust changes in the immune profiles in animal models (Dunphy--Doherty et al., 2018; Scotti et al., 2015; Tuchscherer et al., 2004) and humans (Cole et al., 2015; Jaremka et al., 2013). In a rodent model of maternal postnatal social stress, changes in cytokines, such as ICAM-1 which is involved in inflammation, were reduced in stress exposed dams only when mothers were socially isolated as opposed to provided with social support. This highlights the importance of social interactions during the postnatal period in suppressing the effects of chronic stress and depression (Pittet et al., 2019). Isolation may interact with other social and non-social stressors to adversely impact the immune system (Mattos dos Santos, 2020) and mediate resilience to stress-induced maladies (Dudek et al., 2019), including depression, and this may be particularly relevant during the present Covid-19 pandemic. As we do not have direct data on social support, it is possible that the adverse effects of ethnic stress may have been mediated by social isolation in the present population; mothers separated from their extended family and related community and are likely to experience low levels of support in general (Coburn et al., 2016).

Our results need to be considered in the context of somestudy limitations. Importantly, we could not control for cell-type heterogeneity within the whole blood samples from which the DNA methylation levels were analysed. Physiological immune changes during pregnancy and/or acute inflammation processes at the time of blood drawn could shift the ratio of immune cells which could partially account for the methylation level changes observed at both *FOXP3* TSDR and *TNF-α* promoters. We measured *FOXP3* TSDR and *TNF-α* promoter methylation at the prenatal stage, which are only two markers of a complex immune system that changes across gestation. A comprehensive immune analysis of cells and inflammatory markers across gestation and into the postnatal period would be important to understand whether DNA methylation changes might stem from either acute immune alterations shifting leukocyte cell type ratios, or from chronically altered DNA methylation levels within specific cells. Finally, pre-pregnancy levels of various psychological stressors and associated persistent changes in DNA methylation are largely unknown.

Strengths of our study were the relatively large cohort of Latina pregnant women enrolled during the first half of pregnancy and followed through to the postnatal period and utilization of validated psychosocial instruments and detailed questionnaires. Future studies of other ethnic groups are also needed, as are larger studies that can evaluate what types of stressors and time-windows may be more important. In addition, interventional studies designed to improve the resilience and psychosocial health of women during pregnancy are needed, including immune based strategies.

In sum, our data indicate that markers for higher inflammation during the prenatal period associate with sensitivity to prenatal

discrimination stress. Vice versa, markers for reduced levels of inflammation suggest a resilience to the prenatal stress. In sum, our results suggest that discrimination stress among Latina women may depend on inflammatory state in regard to adverse health outcomes. Additional understanding of these pathways and interventions to improve the psychosocial health of minority women during pregnancy are needed considering uniquely elevated stress, higher levels of inflammatory markers and diseases and pervasive health disparities in this group.

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CRediT authorship contribution statement

Femke Sluiter: were involved in data collection, processing and/or quality assurance. **Angela C. Incollingo Rodriguez:** Formal analysis, performed the statistical analyses and made figures and tables. All authors contributed to the interpretation of the results. **Benjamin C. Nephew:** designed the study, were involved in data collection, processing and/or quality assurance. **Chris Murgatroyd:** Formal analysis, designed the study, were involved in data collection, processing and/or quality assurance, performed the statistical analyses and made figures and tables, wrote the first draft of the manuscript. **Hudson P. Santos:** designed the study, were involved in data collection, processing and/or quality assurance.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2020.100273.

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