

# Pregnancy alters the circulating B cell compartment in atopic asthmatic women, and transitional B cells are positively associated with the development of allergy manifestations in their progeny

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**Problem:** Maternal atopy is a risk factor for allergy. B cells are poorly studied in reproduction and atopy. We aimed to assess how pregnancy affects B cells in atopic women and whether B cells relate to allergic manifestations in offspring.

**Method of study:** Women with and without atopic asthma, pregnant and non-pregnant were enrolled for the study, and circulating B cells were evaluated by flow cytometry, using CD19, CD27, CD38, IgD, and IgM.

**Results:** Compared to healthy non-pregnant, atopic asthmatic non-pregnant (ANP) women presented increased B cell counts, enlarged memory subsets, less transitional cells, and plasmablasts. Atopic asthmatic pregnant (AP) and healthy pregnant (HP) women showed similarities: reduced B cell counts and percentages, fewer memory cells, especially switched, and higher plasmablast percentages. Transitional B cell percentages were increased in AP women with allergic manifestations in their progeny.

**Conclusion:** Atopic asthmatic non-pregnant women have a distinctive B cell compartment. B cells change in pregnancy, similarly in AP and HP women. The recognition that AP women with allergy in their progeny have a typical immune profile may help, in the future, the adoption of preventive measures to avoid the manifestation of allergic diseases in their newborns.

## KEYWORDS

atopy, B lymphocytes, flow cytometry, gestation, human, risk markers

## 1 | INTRODUCTION

Asthma is a common chronic disease with a high and increasing prevalence worldwide, during the last decades.<sup>1</sup> According to the 2008 ARIA update, the majority of asthmatic patients have allergic rhinitis (AR).<sup>2</sup> The majority of patients with allergic asthma and rhinitis are atopic,<sup>3</sup> being sensitized to environmental allergens.<sup>4</sup>

To protect the fetus from aggressive maternal immune responses, physiological immune tolerance mechanisms toward paternal antigens are established during pregnancy, which can interfere with the atopic condition of the mother.<sup>5</sup> Additionally, the characteristic exuberant

changes in the hormonal milieu observed in pregnancy, particularly in late gestation, are thought to have immunomodulatory capacities, although the effects reported are not always consistent.<sup>6,7</sup> Allergic asthma and rhinitis present different outcomes among pregnant women along the gestational period.<sup>8,9</sup> During pregnancy, asthma course can worsen, improve, or remain unchanged.<sup>10,11</sup> Moreover, women presenting with more severe asthma before pregnancy seem to be more likely to worsen symptoms during pregnancy,<sup>5</sup> with exacerbations occurring more frequently in the second trimester of gestation.<sup>10,12</sup> After parturition, however, disease course seems to normalize, returning the before-pregnancy status.<sup>12</sup> Immunologic

mechanisms such as cellular activation, exposure to fetal antigens, or infections, as well as endocrine modulation, emotional stress, fetal gender, or maternal obesity, have been considered as possible causes to justify modifications in the course of allergic diseases from pregnancy until postpartum.<sup>5,13</sup>

For many years, a simplified approach considering the Th1/Th2 paradigm has been proposed for explaining the underlying mechanisms of allergic diseases.<sup>14</sup> Nowadays, many other cellular compartments have been proving their role, such as Th17 cells, regulatory T cells (Tregs), mast cells, and more lately B cells.<sup>15</sup> It is well accepted that Th2 cells induce B lymphocytes to produce allergen-specific IgEs. Increased B cell counts have been described in allergy patients, namely asthmatics, and immature B cells in the first month of age have been related to the development of allergy in young children.<sup>16,17</sup> Nonetheless, the role of B cells in asthma and atopy is poorly understood. Furthermore, alterations in the circulating T cell compartment have been reported in both healthy and atopic pregnant women.<sup>16,18-20</sup> There is also evidence in the literature on the modulation of B cells and their functions along pregnancy, as we have previously described for CD24<sup>Hi</sup>CD38<sup>Hi</sup> regulatory B cells in healthy and atopic asthmatic pregnant (AP) women, but data on the circulating B cell compartment during gestation are scarce.<sup>16,19,21-23</sup>

Apart from the impact of pregnancy on the course of allergic diseases in the mother, maternal history of atopy represents also a risk factor for the development of atopy in the child, even more important than paternal history of atopy.<sup>24</sup> Thus, although the transmission of a more Th2-prone genetic background should be highlighted, it seems that the immunomodulation observed during pregnancy can also affect the developing fetal immune system.<sup>25</sup>

Therefore, we sought to characterize circulating B cells in atopic asthmatic women to further evaluate the impact of pregnancy on this compartment, as these cells have been neglected in reproductive immunology and atopy.<sup>26,27</sup> Moreover, we evaluated the presence of allergy in the progeny of atopic asthmatic women to assess whether its presence could be associated with the characteristics of maternal circulating B cells during pregnancy.

## 2 | MATERIALS AND METHODS

### 2.1 | Patient samples

Women of reproductive age, healthy and asthmatic, pregnant or non-pregnant, followed at CUF Descobertas Hospital (Lisbon, Portugal), were recruited after written informed consent.

Only asthmatics with rhinitis (both clinically diagnosed according to current international guidelines<sup>1,2</sup>) and with proven atopy were included. Atopy was considered as the presence of sensitization to aeroallergens documented by skin prick tests and/or specific IgE. Moreover, for asthma control, asthmatic women were under anti-inflammatory therapy (low median dose of inhaled corticosteroid (ICS)—200–400 µg beclomethasone/daily, and/or long-acting beta-agonists (LABA) and antileukotrienes). Controlled asthma was an inclusion criteria for asthmatic pregnant and non-pregnant

women, and the control of asthma symptoms was evaluated according to GINA guidelines.<sup>1</sup> No asthma exacerbations occurred for at least 6 weeks before sample collection.

All women, according to the eligibility criteria, were included in one of the following groups: AP women, atopic asthmatic non-pregnant (ANP) women, healthy pregnant (HP) women, and healthy non-pregnant (HNP) women.

Pregnant women had uncomplicated, singleton pregnancies. Considering that asthma exacerbations occur mostly in the second trimester of gestation, both groups of pregnant women (AP and HP) were recruited within the third trimester of gestation (31st–36th weeks of gestation), to better exclude possible complications.

Exclusion criteria comprised the following: diabetes; hypertension; autoimmune or any active infectious disease including hepatitis and HIV; any other allergic or respiratory disease; and smoking for the previous 6 months before sample collection. Prenatal use of any medication (other than vitamins, folic acid, and iron supplements) was also an exclusion criterion for HP.

All pregnant women were followed till 6 months after delivery. All newborns were clinically evaluated at 6 months of age. The presence of eczema, recurrent wheeze (≥3 episodes in the first 6 months of age), and food allergy was evaluated. The presence or absence of these allergic manifestations in the progeny of AP women allowed a further division in AP women with allergic manifestations in progeny (AMP) and AP women without allergy manifestations in progeny (NAMP).

All investigations were conducted according to the Declaration of Helsinki. CUF Descobertas Hospital and NOVA Medical School Ethics Committees approved the study.

### 2.2 | Flow cytometry

Peripheral blood was analyzed in a four-color BD FACSCalibur flow cytometer (BDBiosciences, San Jose, CA, USA). BD IMK Kit with BD Trucount™ Tubes (BDBiosciences) was used for B cell enumeration, and B cell subsets were characterized using a panel of monoclonal antibodies, including anti-CD19 (clone HIB19, Biolegend, San Diego, CA, USA), anti-CD27 (clone O323, Biolegend), anti-CD38 (clone HIT2, Biolegend), anti-IgD (clone IA6-2, BDPharmingen, San Jose, CA, USA), and anti-IgM (clone G20-127, BDPharmingen).

### 2.3 | Data analysis

Combined Boolean gating strategies were used for the identification of distinct subsets with Multiset™ and CellQuestPro™ software (BD Biosciences, San Jose, CA, USA). For the IgD/CD27 classification (Figure 1), B cells were divided into subsets: naïve, IgD<sup>+</sup>CD27<sup>-</sup>; unswitched memory/marginal zone like (MZ), IgD<sup>+</sup>CD27<sup>+</sup>; switched memory, IgD<sup>-</sup>CD27<sup>+</sup>; and double-negative (DN) memory, IgD<sup>-</sup>CD27<sup>-</sup>. CD38 and IgM were used to assess transitional B cells (CD38<sup>Hi</sup>IgM<sup>Hi</sup>) and plasmablasts (CD38<sup>Hi</sup>IgM<sup>-/+</sup>).<sup>28</sup> The Bm1-5 classification evaluated the following: transitional (Bm2': IgD<sup>+</sup>CD38<sup>Hi</sup>), naïve (Bm2: IgD<sup>+</sup>CD38<sup>+</sup>), unswitched memory (Bm1: IgD<sup>+</sup>CD38<sup>-</sup>), switched

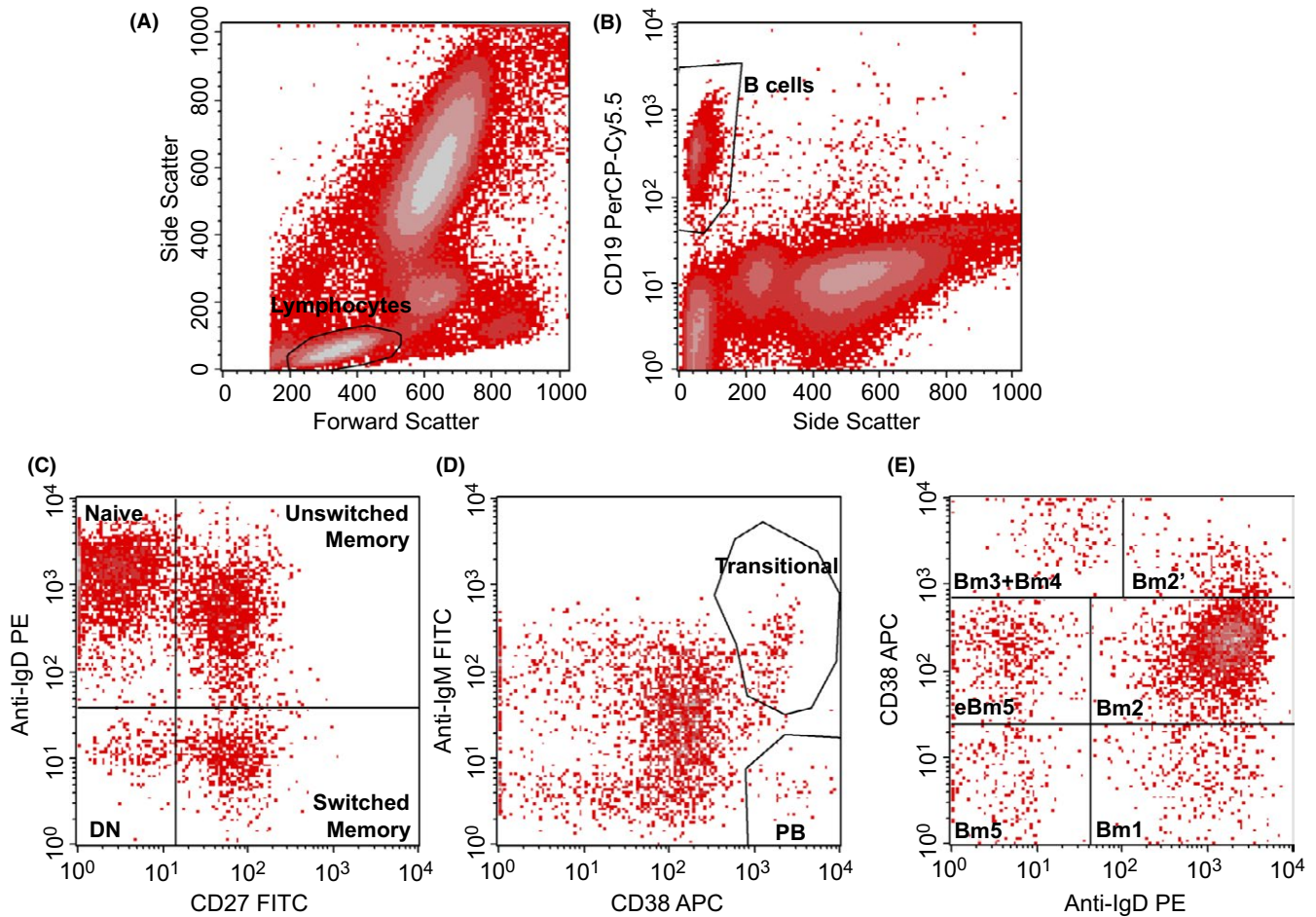


**FIGURE 1** Gating strategy for the identification of distinct B cell subsets. (A and B) Lymphocytes were identified according to forward and side-scatter characteristics (A). B cells were identified as CD19<sup>+</sup> cells (B) within the lymphocyte gate. (C and D) B cell subsets were identified according to the expression of IgD and CD27 in naive, IgD<sup>+</sup>CD27<sup>+</sup>; unswitched memory/marginal zone like (MZ), IgD<sup>+</sup>CD27<sup>+</sup>; switched memory, IgD<sup>+</sup>CD27<sup>+</sup>; and double-negative (DN) memory B cells: IgD<sup>+</sup>CD27<sup>+</sup> (C). Transitional B cells (IgM<sup>hi</sup>CD38<sup>hi</sup>) and plasmablasts - PB (IgM<sup>+</sup>CD38<sup>hi</sup>) were identified according to the expression of IgM and CD38. (E) Bm1-5 classification of B cells according to the expression of IgD and CD38 (Bm1: IgD<sup>+</sup>CD38<sup>+</sup>; Bm2: IgD<sup>+</sup>CD38<sup>+</sup>; Bm2': IgD<sup>+</sup>CD38<sup>hi</sup>; Bm3 + Bm4: IgD<sup>+</sup>CD38<sup>hi</sup>; eBm5: IgD<sup>+</sup>CD38<sup>+</sup>; and Bm5: IgD<sup>+</sup>CD38<sup>+</sup>)

memory (IgD<sup>+</sup>CD38<sup>+</sup>) subsequently divided into post-germinal memory (early eBm5: IgD<sup>+</sup>CD38<sup>+</sup>) and resting memory (late Bm5: IgD<sup>+</sup>CD38<sup>+</sup>) B cells, and plasmablasts (Bm3 + Bm4: IgD<sup>+</sup>CD38<sup>hi</sup>).<sup>29</sup>

## 2.4 | Statistical analysis

Data were analyzed using GraphPad Prism, version 6.01 for Windows (GraphPad Software, La Jolla, California). Statistical significance was defined by *P*-value < .05.

Categorical variables were analyzed with chi-square or Fisher's exact test. For continuous variables, normality was assessed by Shapiro-Wilk test. Multiple groups comparison was performed with ANOVA or Kruskal-Wallis test, followed by Tukey or Dunn multiple comparison tests, whenever *P* < .05. Regarding immune parameters, each two independent groups were compared using Student's *t* test or Mann-Whitney *U* test.

Receiver operating characteristic (ROC) curve evaluation was used to assess the ability of immune parameters to discriminate between asthmatic pregnant women with and without atopic manifestations in

the progeny (using area under the curve [AUC], to estimate the accuracy of discrimination), followed by the application of Fisher's exact test to contingency tables.

Association between B cell subsets and atopic manifestations in the progeny of AP was completed with logistic regression analysis with SPSS Statistics, version 23 (IBM Corp, Armonk, NY, USA).

## 3 | RESULTS

### 3.1 | Patient and sample characteristics

Demographic and anthropometric data of the 134 women included in the study are summarized in Table 1. Body mass index presented significant differences among groups, with HP and AP women showing significantly higher values than HNP and ANP (*P* ≤ .0014; Dunn multiple comparison test), but no differences were observed when comparing AP and HP. Considering parity, HNP women seem to have less nulliparous women than all other groups, but this distribution is not statistically different among HP, ANP, and AP women.

**TABLE 1** Demographic and anthropometric characterization of the groups of women recruited

|                                                         | AP<br>(n=24) | ANP<br>(n=32) | HP<br>(n=43) | HNP<br>(n=35) | P-value             |
|---------------------------------------------------------|--------------|---------------|--------------|---------------|---------------------|
| Maternal age (y), median (IQR)                          | 34 (4)       | 36 (6)        | 32 (5)       | 35 (4)        | .0633 <sup>a</sup>  |
| Ethnicity, n (%)                                        |              |               |              |               |                     |
| Caucasian                                               | 24 (100.0)   | 32 (100.0)    | 42 (97.7)    | 35 (100.0)    | .5454 <sup>b</sup>  |
| Black                                                   | 0 (0.0)      | 0 (0.0)       | 1 (2.3)      | 0 (0.0)       |                     |
| BMI in kg/m <sup>2</sup> , median (IQR)                 | 26.2 (4.5)   | 21.9 (5.0)    | 25.6 (4.1)   | 21.1 (2.4)    | <.0001 <sup>a</sup> |
| Parity, n (%)                                           |              |               |              |               |                     |
| Nulliparous                                             | 12 (50.0)    | 13 (40.6)     | 24 (55.8)    | 5 (14.3)      | .0017 <sup>b</sup>  |
| Multiparous                                             | 12 (50.0)    | 19 (59.4)     | 19 (44.2)    | 30 (85.7)     |                     |
| Education, n (%)                                        |              |               |              |               |                     |
| Basic/high school                                       | 2 (8.3)      | 5 (16.1)      | 4 (9.3)      | 7 (2.3)       | .2956 <sup>b</sup>  |
| Higher education                                        | 22 (91.7)    | 26 (83.9)     | 39 (90.7)    | 23 (76.7)     |                     |
| Smoke exposure during pregnancy, n (%)                  |              |               |              |               |                     |
| Yes                                                     | 3 (12.5)     | –             | 7 (16.3)     | –             | 1.0000 <sup>d</sup> |
| No                                                      | 22 (91.7)    | –             | 36 (83.7)    | –             |                     |
| Gestational age (complete wk) at evaluation, mean (SD)  | 33 (0.9)     | –             | 33 (1.1)     | –             | .2647 <sup>c</sup>  |
| Gestational age (complete wk) at parturition, mean (SD) | 39 (1.1)     | –             | 39 (1.1)     | –             | .2959 <sup>c</sup>  |
| Mode of delivery, n (%)                                 |              |               |              |               |                     |
| Vaginal                                                 | 13 (54.2)    | –             | 18 (41.9)    | –             | .4443 <sup>d</sup>  |
| Cesarean                                                | 11 (45.8)    | –             | 25 (58.1)    | –             |                     |
| Weight at birth in grams, mean (SD)                     | 3262 (329)   | –             | 3199 (402)   | –             | .4931 <sup>c</sup>  |
| Gender, n (%)                                           |              |               |              |               |                     |
| Male                                                    | 12 (50.0)    | –             | 21 (48.8)    | –             | 1.0000 <sup>d</sup> |
| Female                                                  | 12 (50.0)    | –             | 22 (51.2)    | –             |                     |
| Breast feeding, n (%)                                   |              |               |              |               |                     |
| Yes                                                     | 23 (95.8)    | –             | 33 (76.7)    | –             | .0816 <sup>d</sup>  |
| No                                                      | 1 (4.2)      | –             | 10 (23.3)    | –             |                     |
| Sensitization to aeroallergens, n (%)                   |              |               |              |               |                     |
| <i>D. pteronyssinus</i>                                 | 15 (62.5)    | 21 (65.6)     | –            | –             | 1.0000 <sup>d</sup> |
| <i>D. farinae</i>                                       | 12 (50.0)    | 18 (56.3)     | –            | –             | .7875 <sup>d</sup>  |
| <i>Lepidoglyphus destructor</i>                         | 2 (8.3)      | 9 (28.1)      | –            | –             | .0925 <sup>d</sup>  |
| Grass pollens                                           | 25 (20.8)    | 14 (43.8)     | –            | –             | .0924 <sup>d</sup>  |

AP, atopic asthmatic pregnant women; ANP, atopic asthmatic non-pregnant women; BMI, body mass index; HP, healthy pregnant women; HNP, healthy non-pregnant; IQR, interquartile range; SD, standard deviation.

<sup>a</sup>Kruskal-Wallis test, <sup>b</sup>chi-square test, <sup>c</sup>unpaired Student's *t* test, <sup>d</sup>Fisher's exact test. Statistically significant results are indicated in bold.

The division of AP women in two groups: (with or without allergy in the progeny) revealed only a difference in baby gender, with more male babies among AP women with atopic manifestations (Table 2).

### 3.2 | B cell compartment in non-pregnant women: atopic non-pregnant (ANP) vs healthy non-pregnant (HNP)

Compared to HNP (Table 3 and Fig. S1), ANP women presented higher lymphocyte absolute counts ( $P=.0032$ ), with higher B cell counts as

well ( $P=.0007$ ). No differences were observed for B cell percentages (within lymphocytes). In general, all B cell subsets corroborate the trend for significantly higher absolute counts in ANP women. The exceptions were DN B cells (with no alteration of total cell counts in ANP compared to HNP women) and plasmablasts (decreased in ANP women, with a  $P$ -value=.0007). Bm2' and Bm3 + Bm4 cells were the exceptions in the Bm1-5 classification, with the last subset exposing even significantly decreased counts ( $P=.0019$ ) in ANP.

As for percentages (Table 4 and Fig. S1), ANP women presented lower DN B cells ( $P=.0254$ ) and plasmablasts ( $P<.0001$ ). It was possible

**TABLE 2** Demographic and anthropometric comparisons between the subgroups of atopic asthmatic pregnant (AP) women with and without allergy manifestations in the progeny

|                                                               | AP AMP<br>(n=10) | AP NAMP<br>(n=14) | Valor-P                  |
|---------------------------------------------------------------|------------------|-------------------|--------------------------|
| Parity, n (%)                                                 |                  |                   |                          |
| Nulliparous                                                   | 5 (50.0)         | 7 (50.0)          | 1.0000 <sup>b</sup>      |
| Multiparous                                                   | 5 (50.0)         | 7 (50.0)          |                          |
| Education, n (%)                                              |                  |                   |                          |
| Basic/high school                                             | 0 (0.0)          | 2 (14.3)          | .4928 <sup>b</sup>       |
| Higher education                                              | 10 (100.0)       | 12 (85.7)         |                          |
| Smoke exposure during pregnancy, n (%)                        |                  |                   |                          |
| Yes                                                           | 2 (20.0)         | 0 (0.0)           | .1630 <sup>b</sup>       |
| No                                                            | 8 (80.0)         | 14 (100.0)        |                          |
| Gestational age<br>(complete wk) at<br>evaluation, mean (SD)  | 33 (1)           | 33 (1)            | .9524 <sup>a</sup>       |
| Gestational age<br>(complete wk) at<br>parturition, mean (SD) | 39 (1)           | 39 (1)            | .7887 <sup>a</sup>       |
| Mode of delivery, n (%)                                       |                  |                   |                          |
| Vaginal                                                       | 6 (60.0)         | 7 (50.0)          | .6968 <sup>b</sup>       |
| Cesarean                                                      | 4 (40.0)         | 7 (50.0)          |                          |
| Weight at birth in<br>grams, mean (SD)                        | 3279 (177)       | 3249 (411)        | .8122 <sup>a</sup>       |
| Gender, n (%)                                                 |                  |                   |                          |
| Male                                                          | 8 (80.0)         | 4 (28.6)          | <b>.0361<sup>b</sup></b> |
| Female                                                        | 2 (20.0)         | 10 (71.4)         |                          |
| Month of delivery, n (%)                                      |                  |                   |                          |
| Spring/Summer                                                 | 4 (40.0)         | 7 (50.0)          | .6968 <sup>b</sup>       |
| Autumn/Winter                                                 | 6 (60.0)         | 7 (50.0)          |                          |
| Breast feeding, n (%)                                         |                  |                   |                          |
| Yes                                                           | 9 (90.0)         | 14 (100.0)        | .4167 <sup>b</sup>       |
| No                                                            | 1 (10.0)         | 0 (0.0)           |                          |
| Paternal history of atopy, n (%)                              |                  |                   |                          |
| Yes                                                           | 4 (40.0)         | 6 (42.9)          | 1.0000 <sup>b</sup>      |
| No                                                            | 6 (60.0)         | 8 (57.1)          |                          |
| Other family history of atopy, n (%)                          |                  |                   |                          |
| Yes                                                           | 7 (70.0)         | 10 (71.4)         | 1.0000 <sup>b</sup>      |
| No                                                            | 3 (30.0)         | 4 (28.6)          |                          |
| Day-care attendance, n (%)                                    |                  |                   |                          |
| Yes                                                           | 5 (50.0)         | 3 (21.4)          | .2038 <sup>b</sup>       |
| No                                                            | 5 (50.0)         | 11 (78.6)         |                          |

AP AMP, atopic asthmatic pregnant women with allergy manifestations in progeny; AP NAMP, atopic asthmatic pregnant women without allergy manifestations in progeny.

<sup>a</sup>Unpaired Student's *t* test, <sup>b</sup>Fisher's exact test. Statistically significant results are indicated in bold.

to detect significantly higher percentages of Bm1 ( $P=.0126$ ) and Bm5 ( $P=.0057$ ) memory B cells and significantly lower percentages of Bm2' ( $P=.0015$ ) and Bm3 + Bm4 ( $P<.0001$ ) in ANP women.

### 3.3 | B cell compartment in healthy pregnancy: healthy pregnant (HP) vs healthy non-pregnant (HNP) women

Healthy pregnant women presented lower lymphocyte counts, along with lower B cell counts and percentages ( $P\leq.0225$ ), compared to HNP women. Again, all B cell subsets followed the significant decrease in absolute cell counts observed in HP women for total B cells (except unswitched memory B cells).

The B cell compartment in HP women presented lower percentage values of DN B cells ( $P=.0012$ ), transitional B cells ( $P<.0001$ ), and plasmablasts ( $P=.0004$ ). Bm1 ( $P=.0378$ ), Bm2' ( $P<.0001$ ), Bm5 ( $P=.0095$ ), and Bm3 + Bm4 ( $P=.0020$ ) subsets were significantly decreased in HP women, whereas naïve Bm2 cells presented significant higher percentages ( $P<.0001$ ) in these women, compared to HNP women (Tables 3 and 4, and Fig. S2).

### 3.4 | B cell compartment in atopic pregnant (AP) women

Atopic asthmatic pregnant women presented lower absolute counts of total lymphocytes and B cells ( $P<.0001$ ) than ANP women. Significantly lower percentage values of B cells were also observed in AP compared to ANP women ( $P=.0170$ ).

All B cell subsets had decreased cell counts in AP women, except plasmablasts (in both classifications). Switched memory B cell percentages were significantly decreased in AP women ( $P=.0139$ ), but plasmablasts were significantly increased ( $P=.0203$ ) in these women, compared to ANP. Percentages of naïve Bm2 cells were increased in AP women ( $P=.0018$ ), as well as Bm3 + Bm4 ( $P=.0381$ ). On the contrary, Bm1 ( $P=.0296$ ) and Bm5 ( $P=.0020$ ) percentages were significantly decreased in AP when compared to ANP women (Tables 3 and 4, and Fig. S3).

Comparing AP and HP women, no differences were observed for either absolute counts or percentage values other than an increase in the percentages and absolute counts of transitional B cells in AP women (Tables 3 and 4, and Fig. S4).

### 3.5 | B cells of atopic asthmatic pregnant women and allergy in their progeny

Atopic asthmatic pregnant women with allergy in progeny presented lower B cell percentages ( $P=.0240$ ) within total lymphocytes, compared to those without allergy in their progeny. They also showed lower absolute counts of memory ( $P=.0432$ ) and unswitched memory ( $P=.0292$ ) B cells, as well as lower absolute counts of Bm1 cells ( $P=.0316$ ). Identified as the IgM<sup>Hi</sup>CD38<sup>Hi</sup> B cell subset, transitional B cells were significantly increased in AP women with allergy in progeny ( $P=.0012$ ). When identified as the Bm2' subset (Bm1-5 classification), only a nonsignificant trend was observed ( $P=.0558$ ), again with higher values in AP women with allergy in progeny. Results are summarized in Table 5.

The development of allergy in babies from AP was associated with maternal parameters: There was a positive association with

**TABLE 3** Comparison of B cell subsets absolute counts in AP, ANP, HP, and HNP women

|                        |              |               |              |               | Group's comparisons |              |              |          |
|------------------------|--------------|---------------|--------------|---------------|---------------------|--------------|--------------|----------|
|                        |              |               |              |               | ANP vs<br>HNP       | HP vs<br>HNP | AP vs<br>ANP | AP vs HP |
| B cell subsets         | AP<br>(n=24) | ANP<br>(n=32) | HP<br>(n=43) | HNP<br>(n=35) | P-value             | P-value      | P-value      | P-value  |
| Cells/μL, median (IQR) |              |               |              |               |                     |              |              |          |
| Lymphocytes            | 1685 (566)   | 2733 (1301)   | 1753 (701)   | 2081 (866)    | .0032               | .0007        | <.0001       | .9638    |
| B cells                | 188 (126)    | 328 (188)     | 173 (75)     | 259 (129)     | .0007               | <.0001       | <.0001       | .4930    |
| IgD/CD27               |              |               |              |               |                     |              |              |          |
| Naïve                  | 103 (74)     | 188 (141)     | 100 (79)     | 149 (93)      | .0148               | .0016        | <.0001       | .6239    |
| Memory                 | 60 (63)      | 141 (114)     | 64 (41)      | 79 (43)       | .0005               | .0060        | <.0001       | .7727    |
| Unswitched memory      | 38 (38)      | 67 (62)       | 35 (34)      | 45 (35)       | .0003               | .0816        | .0002        | .8736    |
| Switched memory        | 26 (20)      | 64 (39)       | 24 (13)      | 37 (27)       | .0034               | .0001        | <.0001       | .7429    |
| Double-negative        | 6 (5)        | 10 (9)        | 4 (3)        | 9 (8)         | .7665               | <.0001       | .0027        | .2582    |
| Transitional           | 6 (6)        | 11 (7)        | 3 (4)        | 9 (7)         | .1003               | <.0001       | .0002        | .0036    |
| Plasmablasts           | 3 (4)        | 4 (3)         | 2 (1)        | 7 (7)         | .0007               | <.0001       | .3389        | .2710    |
| Bm1-5                  |              |               |              |               |                     |              |              |          |
| Bm1                    | 20 (27)      | 46 (49)       | 12 (13)      | 26 (18)       | <.0001              | <.0001       | <.0001       | .1929    |
| Bm2                    | 115 (81)     | 189 (130)     | 117 (72)     | 149 (91)      | .0045               | .0500        | .0003        | .9044    |
| Bm2'                   | 6 (9)        | 14 (8)        | 4 (5)        | 13 (17)       | .5218               | <.0001       | <.0001       | .0719    |
| Bm3 + Bm4              | 3 (3)        | 4 (4)         | 2 (2)        | 7 (6)         | .0010               | <.0001       | .2022        | .4933    |
| eBm5                   | 22 (13)      | 42 (29)       | 19 (13)      | 27 (23)       | .0019               | .0092        | <.0001       | .9353    |
| Bm5                    | 9 (13)       | 30 (30)       | 8 (6)        | 16 (11)       | <.0001              | <.0001       | <.0001       | .3982    |

AP, atopic asthmatic pregnant women; ANP, atopic asthmatic non-pregnant women; HP, healthy pregnant women; HNP, healthy non-pregnant women; IQR, interquartile range.

Mann-Whitney nonparametric *U* test was used for group's comparison. Results are presented as medians and interquartile range, median (IQR). Statistically significant results are indicated in bold.

transitional B cells (%) and a negative association with total B cells (%) and with absolute counts of memory, unswitched memory, and Bm1 B cells, as displayed in Table 6. Transitional B cell percentage values stood out among these parameters. In the ROC curve of transitional B cell percentages, using the optimal cutoff of 3.59%, the AUC was 0.88 [95% CI: 0.72-1.04], with 85.7% specificity and 90.0% specificity, indicating predictive ability of this parameter to discriminate between AP women with and without allergy in progeny.

Considering the existence of more male babies in the group of AP women with allergy in progeny, we compared transitional B cells from women (AP and HP) with male and female babies to assess a possible relation with baby gender. Neither AP nor HP women presented significant differences in transitional B cells when comparing women with female babies to women with male babies ( $P=.4037$  for AP;  $P=.8461$  for HP).

## 4 | DISCUSSION

B cells have been considered as sole antibody-producing cells, but recently distinct subsets and phenotypes have been reported, with functions in many cases parallel to antibody production.<sup>27</sup> To our

knowledge, this is the first characterization of circulating B cell subsets, in third-trimester AP women, evaluating also its impact as risk factors for the presence of allergy in their progeny. According to our data, ANP women present increased B cell counts, with a more prominent compartment of memory B cells, over a less evident presence of transitional B cells and plasmablasts. In the third trimester of pregnancy, however, the B cell compartment of AP women is somehow similar to the one observed in HP women, both groups experiencing significant decreases in absolute cell counts, with AP women presenting more transitional B cells. Moreover, regarding the progeny of AP women, a more pronounced transitional B cell subset in the mother during the third trimester of pregnancy is associated with the development of allergy during the first 6 months of age.

Controversial results have been reported considering lymphocyte subsets in asthmatic patients.<sup>16,30-32</sup> In our study, absolute lymphocytosis was observed in ANP women, with a consequent elevation of total B cell counts, which were previously described in asthmatic non-pregnant women<sup>16</sup> and atopic children.<sup>32</sup>

The Bm1-5 classification revealed variances between B cells of ANP and HNP women, although few differences were detected with the IgD/CD27 evaluation. Circulating naïve B cells are the major population in both groups of women; however, in ANP, Bm1 and

**TABLE 4** Comparison of B cell subsets percentages in AP, ANP, HP, and HNP women

| B cell subsets                  | AP<br>(n=24)  | ANP<br>(n=32) | HP<br>(n=43)  | HNP<br>(n=35) | Group's comparisons |              |              |             |
|---------------------------------|---------------|---------------|---------------|---------------|---------------------|--------------|--------------|-------------|
|                                 |               |               |               |               | ANP vs<br>HNP       | HP vs<br>HNP | AP vs<br>ANP | AP vs<br>HP |
|                                 |               |               |               |               | P-value             | P-value      | P-value      | P-value     |
| Percentages, median (IQR)       |               |               |               |               |                     |              |              |             |
| Lymphocytes (within leukocytes) | 19.07 (4.50)  | 33.13 (6.45)  | 18.75 (6.15)  | 32.41 (8.31)  | 0.7153              | <0.0001      | <.0001       | .3856       |
| B cell (within lymphocytes)     | 10 (5)        | 12 (4)        | 10 (4)        | 12 (4)        | .2657               | .0225        | .0170        | .6546       |
| IgD/CD27 (within B cells)       |               |               |               |               |                     |              |              |             |
| Naïve                           | 59.14 (22.03) | 55.32 (15.00) | 58.59 (17.80) | 58.81 (20.20) | .4206               | .9761        | .3854        | .8079       |
| Memory                          | 36.50 (19.64) | 40.77 (13.31) | 39.56 (19.03) | 37.79 (17.88) | .2796               | .6103        | .3192        | .6448       |
| Unswitched memory               | 17.86 (13.94) | 22.66 (9.41)  | 23.03 (16.45) | 19.85 (8.17)  | .1920               | .1927        | .6249        | .6542       |
| Switched memory                 | 13.70 (8.43)  | 16.71 (7.84)  | 13.64 (6.78)  | 16.65 (11.86) | .6556               | .1199        | .0139        | .7285       |
| Double-negative                 | 2.85 (1.91)   | 2.31 (2.78)   | 2.54 (1.97)   | 3.87 (1.86)   | .0254               | .0012        | .6076        | .3783       |
| Transitional                    | 3.20 (2.49)   | 2.98 (2.85)   | 1.87 (1.58)   | 3.74 (2.58)   | .3504               | <.0001       | .6076        | .0017       |
| Plasmablasts                    | 1.48 (1.71)   | 1.12 (0.80)   | 1.39 (1.41)   | 2.85 (2.33)   | <.0001              | .0004        | .0203        | .5393       |
| Bm1-5 (within B cells)          |               |               |               |               |                     |              |              |             |
| Bm1                             | 11.05 (8.10)  | 13.77 (10.30) | 7.99 (5.54)   | 10.35 (5.41)  | .0126               | .0378        | .0296        | .1449       |
| Bm2                             | 64.08 (10.56) | 56.00 (14.45) | 67.68 (12.34) | 57.35 (13.12) | .6738               | <.0001       | .0018        | .2492       |
| Bm2'                            | 3.27 (4.25)   | 3.55 (2.66)   | 2.64 (2.76)   | 6.22 (6.37)   | .0015               | <.0001       | .5188        | .1659       |
| Bm3 + Bm4                       | 1.41 (1.92)   | 1.07 (0.85)   | 1.54 (1.44)   | 2.89 (2.22)   | <.0001              | .0020        | .0381        | .9560       |
| eBm5                            | 9.64 (7.26)   | 11.27 (5.89)  | 10.65 (6.06)  | 10.47 (7.40)  | .6288               | .8987        | .0785        | .3118       |
| Bm5                             | 5.67 (3.81)   | 8.83 (7.16)   | 4.69 (3.22)   | 6.00 (3.47)   | .0057               | .0095        | .0020        | .2372       |

AP, atopic asthmatic pregnant women; ANP, atopic asthmatic non-pregnant women; HP, healthy pregnant women; HNP, healthy non-pregnant women; IQR, interquartile range.

Mann-Whitney nonparametric *U* test was used for group's comparison. Results are presented as medians and interquartile range, median (IQR). Statistically significant results are indicated in bold.

Bm5 memory subsets were increased, and on the contrary transitional (Bm2') and plasmablasts (Bm3 + Bm4) were decreased. The rise of memory subsets may be caused by an increased presence of allergen-specific memory B cells (IgE<sup>+</sup>, but mainly IgE<sup>-</sup>) triggered by either systemic or mucosal sensitization, as observed in AR patients.<sup>33</sup> The decrease of circulating plasmablasts in ANP women potentially corroborates a preferential differentiation toward memory B cells in women with controlled asthma. However, animal models of asthma presented evidence of B cell migration toward the lung,<sup>34</sup> and also, distinct homing profiles with impaired circulating plasmablasts have been reported in other pathologies with altered humoral responses.<sup>35</sup> Thus, further studies regarding target organs of asthma and rhinitis are necessary to complete and clarify our data on a different homing pattern vs an altered differentiation in asthmatics.

Resemblances between atopic and healthy pregnant women have been recently described for CD24<sup>Hi</sup>CD38<sup>Hi</sup> regulatory B cells. These cells seem to be modulated similarly in atopic and healthy women from the third trimester of pregnancy to after puerperium.<sup>19</sup> In the present study, regarding the maturation profile of B cells, AP and HP women present similar results during pregnancy, but contrast with those observed in ANP women. Third-trimester pregnant women, including atopic women with controlled asthma (AP), presented decreased

absolute counts of B cells and their respective subsets. Pregnancy-associated B cell lymphopenia has been reported in animal models.<sup>21</sup> Several events can contribute to this decrease in late pregnancy. Animal models show extensive migration of B cells, among other immune cells, into the uterus in late stages of gestation, due to altered chemokine expression patterns.<sup>36</sup> Estradiol, a pregnancy-associated hormone that peaks in the third trimester, has also been considered relevant for B cell modulation, through its negative effect on IL-7.<sup>37</sup> Decreased IL-7 availability during pregnancy arrests B lymphopoiesis.<sup>38</sup> At the same time, progesterone, another hormone peaking in the last weeks of gestation, directly inhibits B cell activation in mice,<sup>39</sup> which can also explain the accumulation of mature, non-activated B cells during pregnancy. In AP women, it seems that the unique environment of pregnancy somehow overlaps the effects of asthma on B cells, as both AP and HP women show substantial similarities in this circulating compartment.

Evaluating therapeutic naïve patients would be ideal for immune characterization. However, use of preventive therapeutics is recommended for pregnant asthmatic women, and so, to better understand AP, ANP women should be as similar as possible to the pregnant group. Nonetheless, therapeutics such as ICS, bronchodilators, or antihistamines, present no consistent effect on lymphocyte

**TABLE 5** Comparison of B cell subsets of atopic asthmatic pregnant (AP) women with (AP AMP) and without (AP NAMP) allergy manifestations in their progeny

| B cell subsets                     | AP AMP<br>(n=10) | AP NAMP<br>(n=14) | P-value      |
|------------------------------------|------------------|-------------------|--------------|
| Percentages, median (IQR)          |                  |                   |              |
| Lymphocytes<br>(within leukocytes) | 18.79 (4.48)     | 20.15 (4.22)      | .3937        |
| B cells (within<br>lymphocytes)    | 9 (4)            | 12 (5)            | <b>.0240</b> |
| IgD/CD27 (within B cells)          |                  |                   |              |
| Naïve                              | 64.48 (28.96)    | 57.32 (17.19)     | .4114        |
| Memory                             | 32.66 (25.74)    | 39.19 (16.80)     | .3626        |
| Unswitched<br>memory               | 17.04 (8.13)     | 22.04 (14.91)     | .1688        |
| Switched memory                    | 13.27 (8.91)     | 14.08 (8.98)      | .8983        |
| Double-negative                    | 2.23 (1.18)      | 3.05 (1.58)       | .1911        |
| Transitional                       | 4.27 (1.37)      | 2.25 (1.67)       | <b>.0012</b> |
| Plasmablasts                       | 1.85 (2.22)      | 1.48 (1.45)       | .8975        |
| Bm1-5 (within B cells)             |                  |                   |              |
| Bm1                                | 8.58 (8.53)      | 2.21 (9.50)       | .1202        |
| Bm2                                | 65.47 (16.12)    | 62.91 (10.23)     | .5409        |
| Bm2'                               | 5.74 (4.26)      | 2.32 (2.43)       | .0558        |
| Bm3 + Bm4                          | 1.91 (3.07)      | 1.41 (1.50)       | .6553        |
| eBm5                               | 8.85 (8.39)      | 9.91 (7.02)       | .6504        |
| Bm5                                | 4.45 (3.98)      | 6.49 (2.98)       | .2301        |
| Cells/ $\mu$ L, median (IQR)       |                  |                   |              |
| Lymphocytes                        | 1740 (594)       | 1675 (809)        | .8210        |
| B cells                            | 144 (126)        | 221 (132)         | .1584        |
| IgD/CD27                           |                  |                   |              |
| Naïve                              | 93 (70)          | 124 (80)          | .3328        |
| Memory                             | 50 (30)          | 86 (78)           | <b>.0432</b> |
| Unswitched<br>memory               | 28 (21)          | 56 (40)           | <b>.0292</b> |
| Switched memory                    | 23 (17)          | 30 (25)           | .1764        |
| Double-negative                    | 4 (3)            | 7 (6)             | .0675        |
| Transitional                       | 7 (4)            | 5 (5)             | .2273        |
| Plasmablasts                       | 2 (4)            | 3 (2)             | .2188        |
| Bm1-5                              |                  |                   |              |
| Bm1                                | 12 (17)          | 27 (26)           | <b>.0316</b> |
| Bm2                                | 95 (71)          | 150 (84)          | .1965        |
| Bm2'                               | 8 (6)            | 5 (3)             | .0559        |
| Bm3 + Bm4                          | 2 (5)            | 3 (2)             | .4021        |
| eBm5                               | 13 (18)          | 22 (11)           | .2902        |
| Bm5                                | 7 (6)            | 13 (15)           | .0912        |

AP AMP, atopic asthmatic pregnant women with allergy manifestations in progeny; AP NAMP, atopic asthmatic pregnant women without allergy manifestations in progeny.

Mann-Whitney nonparametric *U* test was used for group's comparison. Results are presented as medians and interquartile range, median (IQR). Statistically significant results are indicated in bold.

subpopulations.<sup>32,40-42</sup> Exceptions are systemic corticosteroids or high doses of ICS or LABA,<sup>32,40-42</sup> none used in our study, for which our results are unlikely to represent a therapy effect. We also recognize that having more pregnancy-associated time points (at least evaluations in the first and second trimesters of pregnancy) would benefit greatly our study, but it would also be very difficult to achieve for both practical (length of the study) and financial reasons. Yet, the time points selected allowed the identification of pregnancy-associated variations, and the recovery observed postpartum. Moreover, the protocol and laboratory and clinical teams were always the same.

In this study, lung function evaluation was not performed in either AP or ANP women, due to ethical reasons, mostly concerning the danger of exposing third-trimester pregnant women to these procedures. The absence of data on respiratory function (FEV1, FVC, FEV1/FVC) represents a limitation of this work; nevertheless, asthma symptoms in all AP and ANP women were evaluated according to GINA guidelines<sup>1</sup> by the same medical doctor (Allergist) to avoid discrepancies in their clinical assessment. Furthermore, as only controlled asthmatic patients were included, our data represent the immune characterization under controlled disease. Future evaluations of uncontrolled patients with asthma exacerbations may clarify the relation between distinct B cell subsets and disease course.

Despite the reduced numbers of women included in the subgroups of AP women after their separation (AMP and NAMP), this study is also relevant considering the risk for allergy development in the progeny of AP women. We were able to demonstrate that B cells were less represented, but above all, more immature in AP women and particularly in AP women with allergy in offspring at the age of 6 months. Unfortunately, only three children from HP women presented allergy (data not shown), which made it inappropriate to statistically compare AP and HP women with risk manifestations in their progeny.

At birth, approximately half of circulating B cells are transitional B cells, which increase until 4 months of age, declining from that moment on.<sup>17,43</sup> Recently, Lundell et al.<sup>44</sup> reported that children with allergic disease at 18 and 36 months of age presented at birth and 1 month after delivery higher values of CD5<sup>+</sup> B cells, which are mostly transitional B cells. In line with these authors, our data suggest that the more immature B cell compartment in children with risk for developing allergy may have deeper roots, settling probably from a more immature B cell compartment already present in their mothers along pregnancy. B cell activation factor (BAFF) modulates B cell maturation, and interestingly, levels at birth seem to be negatively correlated with transitional B cells and with the risk for allergy development.<sup>17</sup> The link between BAFF, B cells, and risk for allergy development is reinforced also by the observations in cord blood and peripheral blood of children up to 36 months of age. Boys present lower BAFF levels and a delayed B cell maturation with more immature B cells than female babies, being widely reported that young boys are more prone to allergic diseases in early childhood than girls.<sup>17</sup> Although hormonal and pulmonary development reasons can contribute to variations in allergic manifestations between sexes at younger ages,<sup>45</sup> immune parameters and particularly B cells seem implicated in this differential scenario. Again, the present study reinforces this idea, presenting more male babies in the group of

**TABLE 6** Immune parameters associated with the development of allergy manifestations at the age of six months in the progeny of atopic asthmatic women

| Parameter                            | OR [95% CI] <sup>a</sup> | P-value <sup>a</sup> | B <sup>b</sup> | ExpB [95% CI] <sup>b</sup> | P-value <sup>b</sup> |
|--------------------------------------|--------------------------|----------------------|----------------|----------------------------|----------------------|
| B cells (%)                          | 8.5 [1.3-9.6]            | <b>.0351</b>         | -.331          | .718 [.513-1.005]          | .053                 |
| Bm1 (cells/μL)                       | 7.2 [1.1-48.0]           | <b>.0472</b>         | -.084          | .919 [.843-1.002]          | .055                 |
| Transitional B cells (%)             | 54.0 [4.2-692.9]         | <b>.0005</b>         | 1.480          | 4.393 [1.416-13.626]       | <b>.010</b>          |
| Memory B cells (cells/μL)            | 12.0 [1.2-122.3]         | <b>.0333</b>         | -.026          | .974 [.948-1.002]          | .065                 |
| Unswitched memory B cells (cells/μL) | 12.0 [1.2-122.3]         | <b>.0333</b>         | -.049          | .953 [.905-1.003]          | .064                 |

OR, odds ratio; CI, confidence interval.

<sup>a</sup>Fisher's exact test. <sup>b</sup>Logistic regression analysis. Statistically significant results are indicated in bold.

AP women with allergy in their progeny, the subgroup of AP women with increased transitional B cells.

In fact, one of the strengths of our study is that the parameters described, particularly transitional B cells, may therefore represent non-invasive markers for the development of allergy in the child. These parameters can be integrated within maternal screening protocols during pregnancy, allowing for an early diagnosis and post-natal prevention, simultaneously extending this to the prenatal period. Nevertheless, allergic diseases arise mostly after 6 months of age, and this can represent a limitation of the present study. For this reason, to complete the present data, we intend to continue monitoring these children for a longer period and also measure B cell modulating cytokines as BAFF and serum levels of immunoglobulins.<sup>17</sup>

In summary, atopic asthmatic women present a characteristic circulating B cell compartment. During pregnancy, this compartment undergoes important modifications, presenting a distribution of subsets similar to healthy pregnant women. The analysis of circulating B cells in AP women allowed the identification of specific changes during pregnancy in women with allergy in progeny, especially in transitional B cells. These findings, in the future, may be used as risk markers for the development of allergy in children from atopic asthmatic women and allow the adoption of preventive measures to avoid the manifestation of allergic diseases in the newborns.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## SUPPORTING INFORMATION

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