

# Complement component C1q as potential diagnostic but not predictive marker of preeclampsia

Chiara Agostinis<sup>1</sup> | Tamara Stampalija<sup>1</sup> | Dionne Tannetta<sup>2</sup> | Claudia Loganes<sup>1</sup> |  
Liza Vecchi Brumatti<sup>1</sup> | Francesco De Seta<sup>1,3</sup> | Claudio Celeghini<sup>4</sup> | Oriano Radillo<sup>1</sup> |  
Ian Sargent<sup>2</sup> | Francesco Tedesco<sup>5</sup> | Roberta Bulla<sup>4</sup>

<sup>1</sup>Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Trieste, Italy

<sup>2</sup>Nuffield Department of Obstetrics and Gynecology, University of Oxford, John Radcliffe Hospital, Oxford, UK

<sup>3</sup>Department of Medicine, Surgery and Health Sciences, University of Trieste, Trieste, Italy

<sup>4</sup>Department of Life Sciences, University of Trieste, Trieste, Italy

<sup>5</sup>IRCCS, Istituto Auxologico Italiano, Milano, Italy

## Correspondence

Roberta Bulla, Department of Life Sciences, University of Trieste, Trieste, Italy.  
Email: rbulla@units.it

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**Problem:** We have previously found that C1q is constitutively expressed by invading trophoblast and endothelial cells of decidua and contributes to vascular and tissue remodeling. Based on these findings, we sought to determine whether there were changes in the circulating level of C1q that may be used as a diagnostic and predictive marker of preeclampsia.

**Method of Study:** We measured the levels of C1q, C4, and complement activation products in serum or plasma of normal pregnant women and preeclamptic patients from different cohorts.

**Results:** We observed a marked decrease in the concentration of C1q associated with a reduced level of C4 in preeclamptic patients as compared to matched healthy pregnant woman but no significant difference in the circulating level of the activating products C5a and the soluble terminal complement complex sC5b-9. Analysis of serum samples collected at early phase of pregnancy from women who later developed preeclampsia failed to show a decrease in C1q level.

**Conclusion:** The results of the present investigation demonstrate that low levels of C1q and C4 are associated with preeclampsia but cannot be used as predictive markers.

## KEYWORDS

C1q, C4, complement components, preeclampsia, syncytiotrophoblast micro- and nanovesicles

## 1 | INTRODUCTION

Preeclampsia (PE) is a multisystemic disorder occurring in pregnancy that manifests with new onset hypertension and proteinuria corrected by removal of the placenta.<sup>1</sup> Failure of invasive EVT to modify the walls of maternal spiral arteries before the mid-second trimester contributes to reduce blood supply to the placenta. The development of PE is also contributed by oxidative stress related to elevated glucose in diabetes or to inflammation.<sup>2</sup> A systemic inflammatory response is widely accepted to be associated with normal pregnancy, particularly during the third trimester and becomes more intense in preeclamptic patients as a result of placental oxidative stress leading to the clinical manifestations of this syndrome.<sup>3</sup> The inflammatory process involves vascular

endothelium, circulating leukocytes and pro-inflammatory cytokines including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and interleukin-8 (IL-8).<sup>4</sup> The release of pro-inflammatory trophoblast debris that increase in PE is considered an important cause of pregnancy-associated inflammation.<sup>5</sup>

The complement system (C) is an important component of the innate immune system and is involved in host defense against infectious agents as well as in the clearance of immune complexes and damaged cells. In addition, C contributes to promote the inflammatory process through the release of the pro-inflammatory peptides C4a, C3a, and C5a, and the non-cytolytic complex C5b-9.<sup>6-10</sup> Normal pregnancy is characterized by a gradual increase in the serum levels of C3, C4, and total complement activity;<sup>11,12</sup> C activation has been

extensively investigated in preeclamptic patients and circulating split products of C components have been reported to be mainly associated with the disease severity. Activation of the alternative pathway has been implicated in the development of PE by Lynch and colleagues<sup>13</sup> following the observation of increased levels of the fragment Bb in preeclamptic patients. There is also evidence that C may be activated through the classical pathway as suggested by the increased level of C4d found in preeclamptic patients by Derzsy et al.<sup>14</sup> The involvement of the lectin pathway in C activation in preeclamptic patients is less clear. Although a higher concentration of mannose-binding lectin (MBL) has been demonstrated in the plasma of patients compared to normal pregnant women,<sup>15</sup> the activity of MBL/MBL-associated serine proteases (MASP)2 complexes is not increased in preeclamptic women.<sup>16</sup> Other groups reported that the association of a genetically related MBL polymorphism with MBL decreased functional activity is protective against PE.<sup>17</sup>

We have focused our research interest on C1q following the observation that this recognition molecule of the classical pathway is abundantly expressed in maternal decidua in normal pregnancy, it is locally produced, and it is involved in the endovascular<sup>18</sup> and interstitial<sup>19</sup> invasion of trophoblast cells. Furthermore, this molecule is involved in angiogenesis<sup>20</sup> and acts as tumor promoting factor<sup>21</sup>. Defective placentation has been observed in C1q knockout mice showing increased fetal resorption frequency and decreased birthweight.<sup>19</sup> Singh and colleagues provided data supporting our observations showing that pregnant C1q-deficient (*C1q*<sup>-/-</sup>) mice recapitulate the key features of human PE such as hypertension, albuminuria, endotheliosis, decreased placental vascular endothelial growth factor (VEGF), and elevated levels of soluble VEGF receptor 1 (sFit-1).<sup>22</sup>

In this study, we sought to investigate whether the local role of C1q in the process of placental development could be reflected at the peripheral level in the pathogenesis of PE. We therefore investigated whether circulating levels of C1q were different in preeclamptic patients compared to healthy women. We also wanted to understand whether these differences could have a predictive significance.

## 2 | MATERIAL AND METHODS

### 2.1 | Subjects

Preeclamptic patients and healthy women were recruited in the Nuffield Department of Obstetrics & Gynaecology (NDOG), Oxford

University (UK), and the Department of Obstetrics & Gynaecology of IRCCS "Burlo Garofolo" (Trieste, Italy).

To determine changes in C components associated with PE, plasma samples were collected using EDTA anticoagulation tubes from women with PE (n=30) who were matched for age, parity, and gestational age to 30 normal pregnant women (Table 1). PE was defined as the new onset of a systolic blood pressure (BP)  $\geq$  140 mm Hg or diastolic BP  $\geq$  90 mm Hg on at least two occasions within 24 hours and new onset proteinuria  $\geq$ 300 mg in a 24-hour urine collection, 50 mg/mmol protein/creatinine ratio, or at least 2+ on dipstick testing on two consecutive measurements. For measurement of blood pressure, the women were rested and reclining at an angle of 45° and a digital device was used. All patients and healthy controls had singleton pregnancies with undetected fetal abnormality. Blood samples were collected, plasma was isolated, and the samples stored at -80°C until analysis. These studies were approved by the Oxfordshire Research Ethics Committee, adhere to the principles of the Declaration of Helsinki, and written consent was obtained from each participant.

PE patients and control pregnant women were recruited in the Trieste's Institute "Burlo Garofolo" following the same criteria described above. Serum samples of 13 women with PE were matched for age, parity, and gestational age to 13 normal pregnant women (Table 2). Non-pregnant women, with age ranging from 20 to 40 years, who had no clinical and laboratory alterations and were not taking hormonal contraceptives, were also enrolled in Trieste's Institute. The cohort of pregnant women for prospective studies was followed by the Prenatal Diagnosis and Gynaecologic Unit of the Institute for Maternal and Child Health, IRCCS "Burlo Garofolo" in Trieste, Italy. All women were recruited from October 2007 to April 2009 as described by G. Di Lorenzo and colleagues.<sup>23</sup> Patients were matched for maternal age (32 $\pm$ 4) and parity (nulliparity 19/25). Patients were followed from first-trimester ultrasound aneuploidy screening to delivery. The study was approved by the Research Ethics Committee of the Institute Burlo Garofolo and adheres to the principles of the Declaration of Helsinki. We enrolled singleton pregnancies between 11 and 13 weeks of gestation. All pregnancies were dated by last menstrual period if consistent with crown-rump length (CRL) measurements ( $\pm$ 7 days). We selected from this serum bank all women (n=25) who subsequently developed preeclampsia and 25 matched control, following the criteria for inclusion described above.

Plasma samples	Normal pregnant (n=30)	Preeclamptic (n=30)	P
Age (y)	29.5 ( $\pm$ 5.5)	30.7 ( $\pm$ 5.8)	n.s.
Nulliparity	22/30	22/30	n.s.
Gestation at sample (d)	238.2 ( $\pm$ 35.6)	241.5 ( $\pm$ 32.5)	n.s.
Booking systolic BP (mm Hg)	109.5 ( $\pm$ 13.0)	140.5 ( $\pm$ 14.4)	P<.05
Booking diastolic BP (mm Hg)	65.9 ( $\pm$ 9.9)	88.5 ( $\pm$ 14.9)	P<.05
BMI	23.9 ( $\pm$ 0.9)	28.0 ( $\pm$ 1.8)	P<.05
Maximum 24-h proteinuria (mg)	<300	4672.7 ( $\pm$ 4721)	P<.05

**TABLE 1** Characteristics of the cohort from Nuffield Department of Obstetrics & Gynaecology (NDOG), Oxford University (UK) (n=60)

Data are expressed as mean values ( $\pm$  standard deviation). n.s., not significant.

**TABLE 2** Characteristics of the cohort from Department of Obstetrics & Gynaecology of IRCCS “Burlo Garofolo” (n=26)

Serum samples	Normal Pregnant (n=13)	Preeclamptic (n=13)	P
Age (y)	29.5 (±3.1)	36.8 (±2.2)	n.s.
Nulliparity	7/13	8/13	n.s.
Gestation at sample (d)	203 (±3.7)	231 (±1.9)	n.s.
Booking systolic BP (mm Hg)	110 (±7.0)	179 (±19.1)	P<.05
Booking diastolic BP (mm Hg)	70 (±6.1)	104 (±9.4)	P<.05
BMI	n.d.	26.2 (±6.4)	n.d.
Maximum 24-hr proteinuria (mg)	<300	2588.1 (±1648.1)	P<.05

Data are expressed as mean values (± standard deviation). n.s., not significant; n.d., not defined.

## 2.2 | Placental perfusion and isolation of syncytiotrophoblast extracellular vesicles (STBM)

STBM were prepared using a dual-placental perfusion system as previously described.<sup>24</sup> Placentas obtained at cesarean section without labor, from healthy and preeclamptic women (Table 3), were processed immediately and, following an equilibration period, were perfused for 3 hour in a closed circuit. At the end of the 3-hr perfusion period, the maternal-side perfusate was centrifuged at 600 g for 10 minutes at 4°C to remove large debris. The supernatant was centrifuged at 150,000×g (maximum) for 1 hour at 4°C to pellet the vesicles. The resultant pellets were pooled and washed in PBS before being resuspended in PBS to give a final protein content of 5 mg/mL, as assessed using a BCA protein assay kit, and stored in aliquots at -80°C until subsequent use.

## 2.3 | Quantitation of C component level

Commercial ELISA kits were used for the measurement of plasma and serum levels of C1q (HyCult, Milan, Italy). C3 and C4 were measured with immunoturbidimetric assays for Roche/Hitachi analyzer (Cobas; Roche, Milan, Italy). Analysis of complement components bound to STBM was performed by Sandwich ELISA as described by Bulla et al.,<sup>25</sup> on vesicles solubilized with Tris buffer saline pH 8, NP40 0.5%, BSA 1 mg/mL, PMSF 1 mol/L (TNNB).

## 2.4 | Quantitation of sC5b-9 and C5a levels by ELISA

The level of C5a was measured using mAb C17/5 as trapping antibody and mAb G25/2 as revealing reagent (both provided by Prof. Oppermann, University Medicine Goettingen, Germany) according to

**TABLE 3** Patient details for normal pregnant (n=4) and preeclamptic women (n=6) STBM

STBM samples	Normal Pregnant (n=4)	Preeclamptic (n=6)	P
Age (y)	33 (±1.8)	35 (±2.4)	n.s.
Nulliparity	0/4	0/6	n.s.
Gestation at sample (d)	273 (±0.6)	259.5 (±9.4)	n.s.
Booking systolic BP (mm Hg)	110 (±1.8)	124.50 (±5.4)	P<.05
Booking diastolic BP (mm Hg)	60 (±2.3)	69.5 (±5.9)	P<.05
BMI	27.2 (±1.5)	27.8 (±3.1)	n.s.
Maximum 24-hr proteinuria (mg)	<300 mg	1306.8 (±999.5)	P<.05

Data are expressed as mean values (± standard deviation). n.s., not significant.

Oppermann et al.<sup>26</sup>. The amount of sC5b-9 was evaluated using solid-phase bound mAb aE11 and biotin-labeled goat IgG anti-C5 followed by alkaline phosphatase conjugate to streptavidin (Sigma-Aldrich Milan, Italy) following published procedures.<sup>7</sup>

## 2.5 | Statistical analysis

C1q data in non-pregnant, normal pregnant women in the three trimesters of pregnancy were analyzed with Kruskal-Wallis test. Nonparametric test (Mann-Whitney) was carried out as data were not normally distributed. To analyze C component levels, the paired t test was used for analysis to compare the paired data. Significance was set at P≤.05. Statistical analyses were carried out using GraphPad Prism version 4.0 (GraphPad Inc., San Diego, CA, USA).

## 3 | RESULTS

### 3.1 | Levels of C1q in normal pregnant women and preeclamptic patients

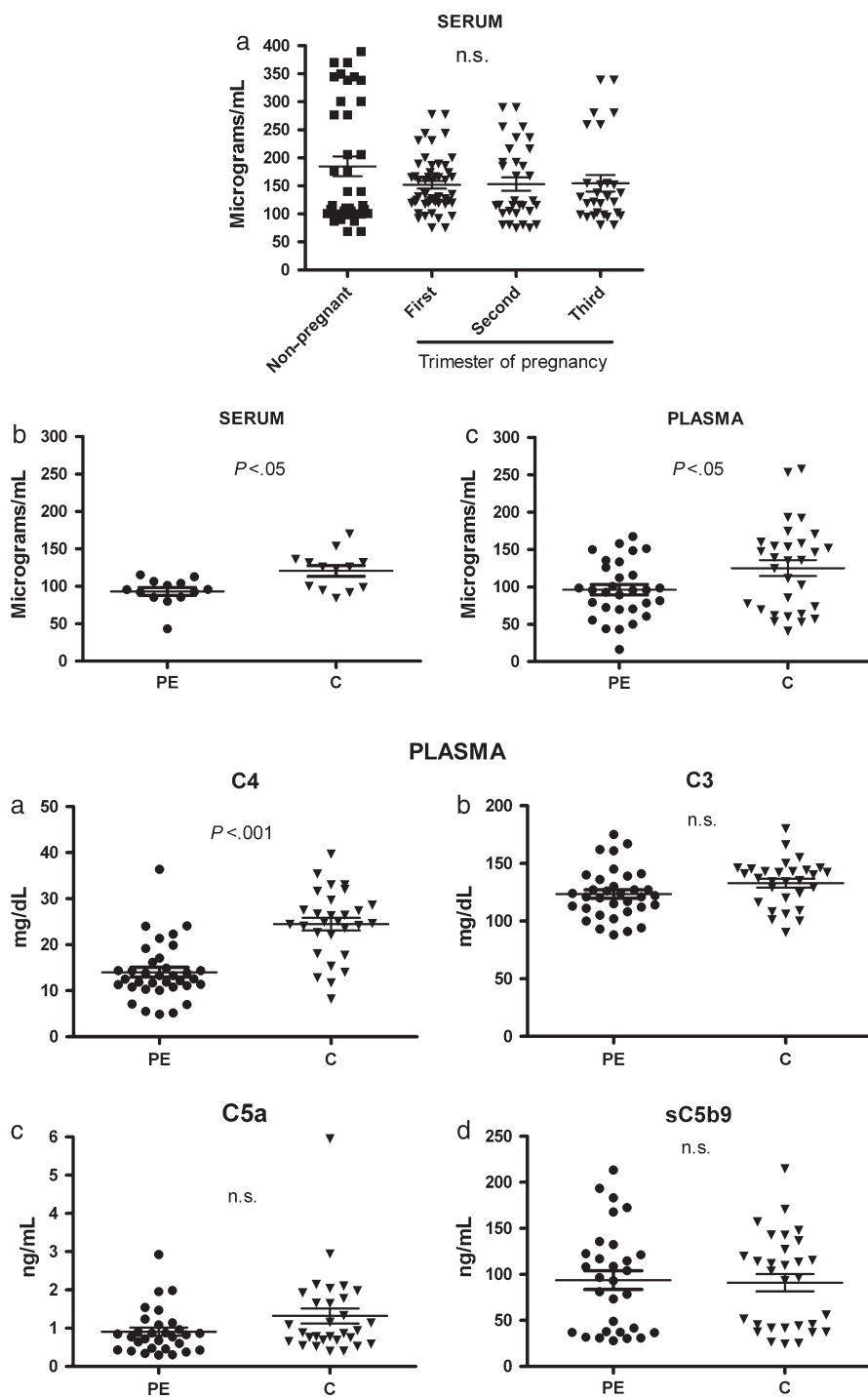
As the level of several C components rises during pregnancy, we measured the concentration of C1q in normal pregnant women in the three trimesters of pregnancy and compare the results with those obtained in non-pregnant controls. As shown in Figure 1a, the levels of C1q in first-trimester pregnant women ranged between 80 and 280 µg/mL with a mean value of 150 µg/mL. These values remained essentially unchanged throughout the pregnancy trimesters and were not significantly different from those observed in non-pregnant controls. We next assessed the concentrations of C1q in preeclamptic patients and in healthy pregnant women and observed significantly

reduced levels in preeclamptic patients as compared with the control group (Figure 1b-c).

### 3.2 | Measurement of C4, C3 and C activation products in preeclamptic patients

To ascertain whether the decreased levels of C1q observed in preeclamptic patients were restricted to this early recognition molecule and did not involve other C components, we measured the level of C4 and C3. The analysis was conducted on plasma samples obtained from 30 preeclamptic and 30 healthy pregnant women attending the Nuffield

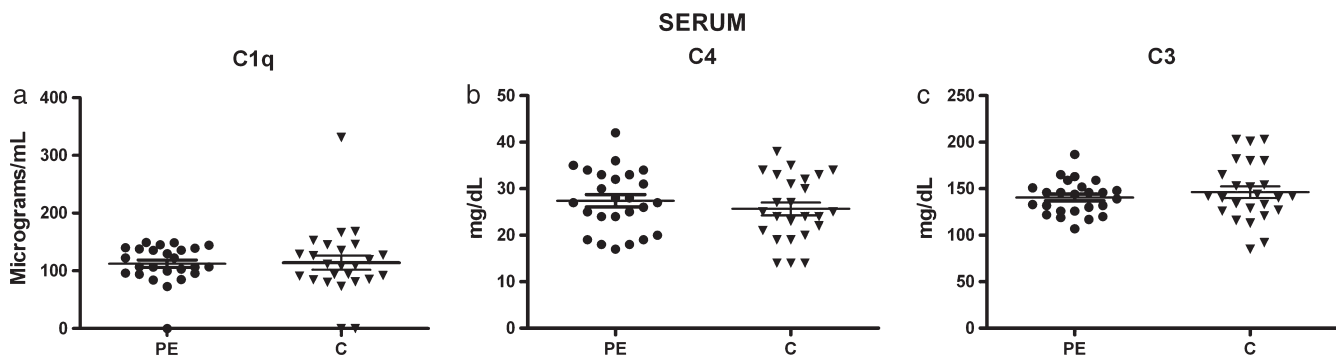
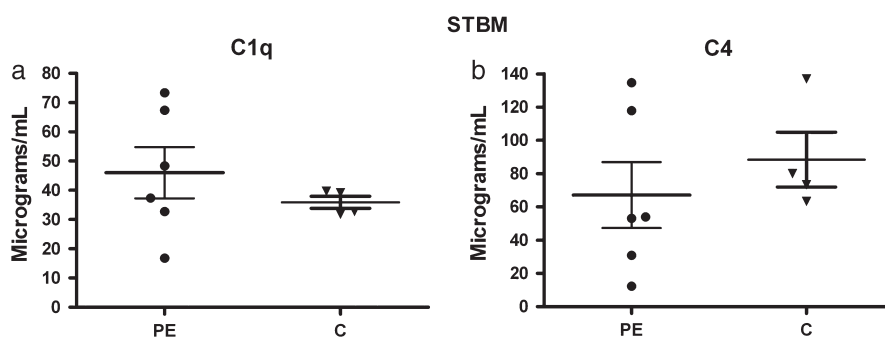
Department of Obstetrics & Gynaecology (NDOG) at Oxford University (Table 1). The reason for using plasma instead of serum was to avoid in vitro C activation that occurs during the preparation of serum as a result of coagulation and fibrinolysis. The results presented in Figure 2a and B revealed a significant decrease in the level of C4 and only a slight decrease in C3 concentration confirming similar data already obtained with C1q (Figure 1c) and suggesting an ongoing activation of the classical pathway. We then searched for circulating activation products of the C-terminal pathway C5a and sC5b-9, which are known to promote the inflammatory process at tissue level. As showed in Figure 2c and d, the data showed that the circulating levels of the activation products



**FIGURE 1** (a) Serum levels of C1q were analyzed in control non-pregnant women (n=38), healthy pregnant women at first (n=50), second (n=32), and third (n=28) trimesters. n.s.= non-significant with Kruskal-Wallis test. (b, c) Measurement of C1q in preeclamptic patients (indicated with "PE") and matched control pregnant women (indicated with "C") enrolled at Trieste's Hospital (b) and Oxford's Hospital (c). In both cohorts, C1q was significantly decreased in preeclamptic women vs controls (*paired t test*). The results are expressed as box plot graphs, in which the line in the middle of the box represents the median; the lower and the upper edges of the box are the 1st and 3rd quartile, respectively

**FIGURE 2** Measurement of C4 (a) and C3c (b), C5a (c), and sC5-b9 (d) in plasma samples of preeclamptic patients (n=30) and correlated control women (n=30) enrolled at Oxford's Hospital. C4 levels resulted significantly lower in preeclamptic patients if compared with healthy matched control women. No differences were observed in term of C3c, C5a, and sC5-b9 levels (*paired t test*). The results are expressed as box plot graphs, in which the line in the middle of the box represents the median; the lower and the upper edges of the box are the 1st and 3rd quartile, respectively

**FIGURE 3** Analysis of C1q (a) and C4 (b) bound to STBM was performed by Sandwich ELISA, solubilizing the microvesicles derived from six preeclamptic and four control placenta. No statistical differences were observed between preeclamptic and control STBM (Mann-Whitney U-test). Bars represent mean  $\pm$  standard error



**FIGURE 4** Serum levels of C1q (a), C4 (b), and C3c (c) measured at first trimester of pregnancy in women with a normal pregnancy (n=25) and women who subsequently developed preeclampsia (n=25). No statistical differences were observed in these groups of samples (*paired t test*). The results are expressed as box plot graphs, in which the line in the middle of the box represents the median; the lower and the upper edges of the box are the 1st and 3rd quartile, respectively

C5a and sC5b-9 did not increase in preeclamptic plasma samples. Furthermore, no correlation has been found between C5a or sC5b-9 levels and proteinuria, blood pressure, and gestational age (data not shown).

### 3.3 | Measurement of C1q and C4, in STBM

As the reduced levels of C1q and C4 were not associated with increased concentration of activation products of the late components, we searched for complement deposits on microvesicles as a possible mechanism of removal of this complement components. STBM circulate in the plasma of pregnant women starting from the late first trimester onwards. To this end, we performed sandwich ELISA assay on microvesicles solubilized with TNNB. The results presented in Figure 3 showed that C1q and C4 were both present on STBM from preeclamptic placentae and from normal placentae with no significant difference. It is important to emphasize that the level of STBM circulating in preeclamptic patients is higher than that observed in normal pregnant women and this may justify the removal of an increased amount of C1q and C4.

### 3.4 | Evaluation of C1q and C4 as predictive markers of PE

Having found markedly decreased levels of the early complement components, we decided to investigate whether differences in the

concentration of these proteins between preeclamptic patients and healthy controls could be used as an early indication of PE with predictive significance. To this end, we quantified C1q, C4, and C3 in 25 sera of pregnant women collected between 11<sup>o</sup> and 13<sup>o</sup> gestational weeks who subsequently developed PE and in 25 sera of healthy pregnant women. These serum samples were obtained from a bank of sera collected for prospective studies from pregnant women followed by the Prenatal Diagnosis and Gynaecologic Unit of the Institute for Maternal and Child Health e IRCCS "Burlo Garofolo" in Trieste, Italy (see section 2). No statistical differences were observed between the two groups in circulating levels of C1q, C4, and C3 (Figure 4).

## 4 | DISCUSSION

In this study, we have evaluated the C1q level in pregnant women as a potential diagnostic and possibly predictive marker of PE and report data indicating that it is markedly reduced in preeclamptic patients compared to age-matched normal pregnant women. The reason that led us to investigate possible changes in the concentration of C1q in these patients was prompted by our initial observation that decidual C1q is critical for tissue and vascular remodeling and that C1q-deficient mice exhibit reduced vascular remodeling, a clinical feature of preeclampsia, and increased fetal resorption rate.<sup>18,19</sup> The importance of C1q on pregnancy outcome is also supported by the finding that pregnant C1q-deficient mice recapitulate the key features

of human PE including hypertension, albuminuria, endotheliosis, decreased placental vascular endothelial growth factor (VEGF), and elevated levels of soluble VEGF receptor 1 (sFlt-1) that correlate with increased fetal death.<sup>22</sup> Based on these findings, we hypothesized that the expression of C1q may be reduced in preeclamptic placentae and may in turn be also accompanied by a decrease in the concentration of C1q in the circulation.

Analysis of C1q levels in pregnant women showed that they remain stable through pregnancy and are similar to that found in sera from non-pregnant women. This observation was rather surprising because previous studies have shown that the level of other C components such as C4, C3, and MBL progressively increase in normal pregnancy.<sup>14</sup> A possible explanation for this different behavior is that C1q, unlike MBL, C4, and C3, is not an acute phase reaction protein and therefore is not influenced by the inflammatory process that is currently believed to be associated with physiologic pregnancy.<sup>27,28</sup>

Our data revealed a marked decrease in the level of C1q in preeclamptic patients compared to the control group. Although a reduced production of C1q that may contribute to the low level cannot be excluded, we believe that an increased consumption due to C activation offers a more plausible explanation. This possibility is supported by the finding that the reduced level of C1q is associated with decreased concentration of circulating C4 suggesting activation of the classical pathway of the C system. One possible explanation for complement activation resulting in C1q consumption is the presence of immune complexes in PE patients. ICs have been detected in the serum of these patients particularly in the third trimester of pregnancy and found to be associated with an increased severity of the clinical manifestations.<sup>12</sup> Complement consumption may also be induced by agonistic autoantibodies to the major angiotensin II type 1 (AT1) receptor that have been detected in PE patients<sup>29</sup> and found to reproduce the key clinical features of PE following injection into pregnant mice.<sup>30</sup> Xia and Kellems et al. have shown that complement is activated by the antibodies bound to AT1 and mediate the development of hypertension and proteinuria in the mouse model through the release of C3a.<sup>31</sup> Activation of the classical pathway involving C1q and C4 has also been documented on placental villi and kidney glomeruli of preeclamptic patients.<sup>32,33</sup>

An alternative possibility is that C1q is removed by binding to cells undergoing apoptosis. Apoptosis has been documented by several groups in the placental villi of preeclamptic patients.<sup>34,35</sup> C1q binds to apoptotic cells through the globular heads and is recognized by phagocytes displaying specific receptors.<sup>36</sup>

An interesting observation of this work is that C1q was found to be deposited on STBM which are released from placenta into the maternal circulation.<sup>37</sup> Although we do not observed a significant difference in C1q binding to normal and preeclamptic STBM, it is important to point out that PE placentas release a substantial higher amount of debris than normal placentas and PE STBM are likely to remove relatively more C1q than control STBM.<sup>38</sup> On the basis of these observations, we can speculate that C1q levels reflect a downstream effect of tissue damage associated with PE rather than an upstream effect.

The C3 level in PE patients was within the range observed in healthy pregnant women confirming data reported by other

groups.<sup>12,14</sup> However, the normal level of C3 does not exclude activation of this C component as suggested by the finding of increased level of C3 activation products including C3d and C3a published in the literature.<sup>12,14</sup> Our failure to detect increased levels of C5a and SC5b-9 in preeclamptic patients was rather surprising because other groups have found a significant increase in circulating activation products of the C-terminal pathway.<sup>14,39-40</sup> Burwick and colleagues<sup>41</sup> have also reported increased levels of urinary C5b-9 in severe PE that were correlated with total urine proteins. The reason for these contrasting results is not apparent and may probably depend on the different study group examined, the time of blood sample collection, and also on the severity of the disease.

One potential weakness of our study is the small sample size and the inclusion of mild preeclampsia subjects that prevent us in performing sensitivity and specificity analyses.

The goal of our prospective study was to investigate whether the lower level of C1q observed in preeclamptic patients could be detected before the onset of the clinical manifestation of the disease and thus be used as a predictive marker of PE. We failed to detect a significant change in C1q level which could be used to predict the development of PE as it has been shown by Lynch and colleagues<sup>13</sup> for the increased level of activated fragment Bb. It may well be that the presence of other risk factors may differently influence the level of C1q and Bb in the early phase of the disease. It is also possible that the activation of the alternative pathway precedes the involvement of C1q and the activation of the classical pathway.

This study clearly shows a significantly decrease in the circulating levels of C1q in PE. The lower concentration of C1q is likely to be associated with its ability to bind to circulating IC and STBM as well as to apoptotic and necrotic blebs in the villi of PE placentae. The fact that the levels of C1q and C4 are both significantly reduced in PE patients suggests an ongoing activation of C through the classical pathway. Finally, the results of the present investigation demonstrate that the levels of C1q and C4 cannot be used as predictive markers of PE.

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