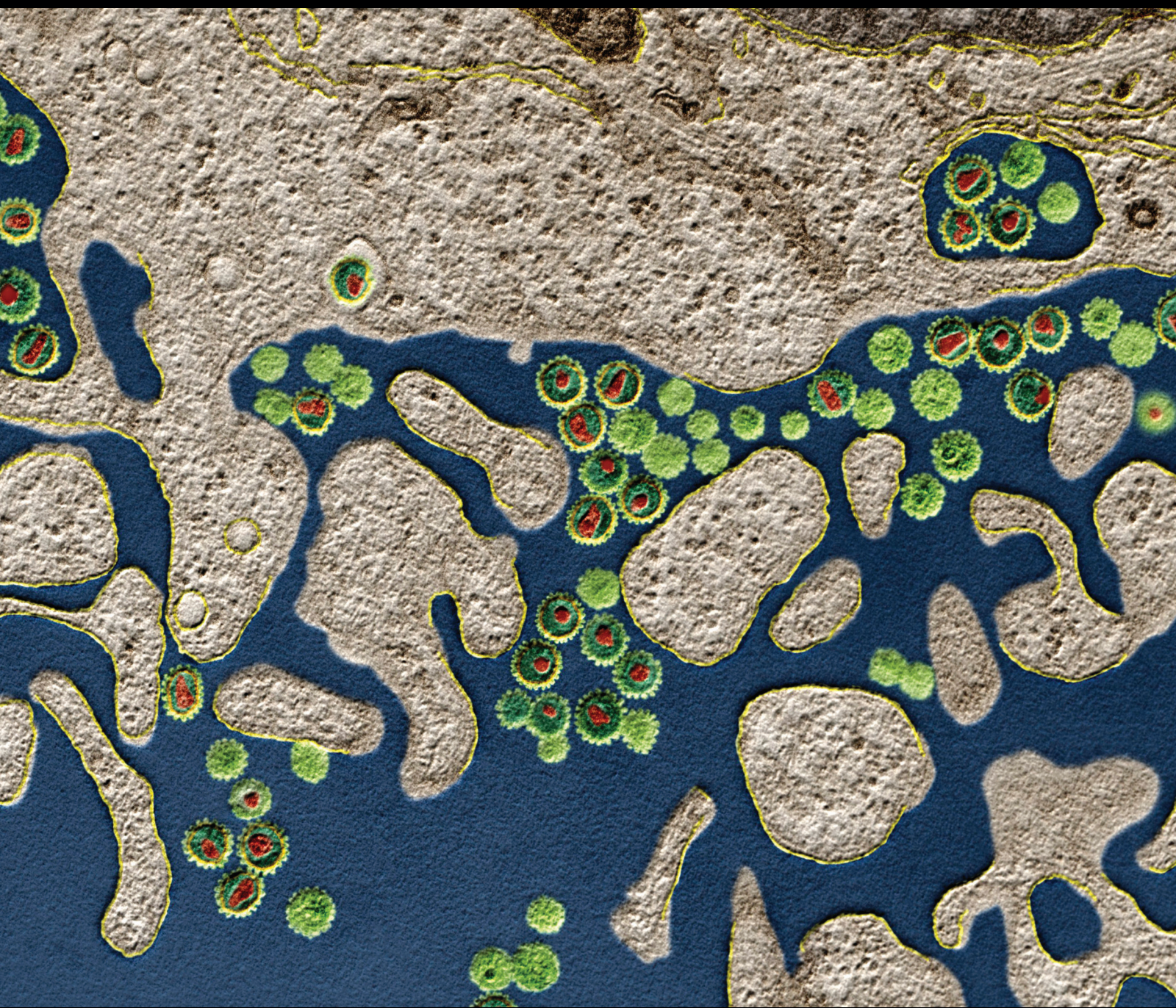
Journal of Immunology Research

# Autoimmunity in Reproductive Health and Pregnancy

Special Issue Editor in Chief: Jacek Tabarkiewicz
Guest Editors: Senthami R. Selvan and Nathalie Cools



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# Editorial Board

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 9501865, 3 pages
https://doi.org/10.1155/2018/9501865

*Editorial*

# Autoimmunity in Reproductive Health and Pregnancy

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Received 2 January 2018; Accepted 2 January 2018; Published 13 February 2018



The influence of pregnancy on the maternal immune system is complex and orchestrated by multiple hormonal and metabolic factors provided by the mother as well as the fetus. The modifications of the maternal immune response include changes in cell proportions, phenotypes, and their functional ability to produce cytokines and other mediators. During pregnancy, activations of pro- and anti-inflammatory responses are fluently regulated depending on the phase of pregnancy [1]. Implantation, placentation, and delivery phases are proinflammatory, while the period of rapid fetal growth and development is anti-inflammatory [1]. The model of "immune suppression" during pregnancy has been long accepted, yet at the present, we are aware that fetal tolerance is not caused by suppression of the maternal immune system but rather by immunomodulation and that pregnant women are very much capable of having robust immune responses [2]. An understanding of the balance between tolerance and protection of the fetus and maternal active immune response against pathogens or self-antigens may contribute to developing new approaches to the problem of autoimmunity in reproductive health and pregnancy.

Autoimmune diseases are characterized by organ and tissue damage caused by self-reactive immune responses mediated by antibodies and/or T cells. These diseases may be associated with genetic and/or environmental predispositions. Thus, autoimmune disorders predominantly affect women and often occur during reproductive years and have implications for fertility and pregnancy to some extent [3, 4]. The relationships between autoimmunity and reproduction include the impact of pregnancy on the clinical course of autoimmune disorders as well as the influence of autoimmunity on pregnancy development. Thus, in this population of patients, specialized concerns in pregnancy planning and management are commonly encountered. Autoimmune diseases are usually thought to be associated with pathogenic activity of Th17-/Th1-type cells; during pregnancy, Th2-type cytokines are noted to be crucial to maintain the tolerance of the mother towards the fetal semiallograft [3, 4]. In pregnancy, immunoregulatory cytokines and cells are present in the mother's circulatory system and accumulate in the decidua and can modify autoimmune responses influencing the symptoms of autoimmune disease.

Systemic lupus erythematosus (SLE), primary antiphospholipid syndrome (APS), and secondary SLE-associated APS affect mostly women of childbearing age [5]. In a paper by Andreoli et al., we found the most current recommendations of the European League Against Rheumatism (EULAR) for women's health issues in SLE and/or APS that were developed using an evidence-based approach followed by an expert consensus [5]. The considerations in the expert consensus were as follows: family planning, reduction of the risks of adverse maternal or fetal outcomes, use of hormonal contraception and menopause replacement therapy, fertility preservation during therapy, assisted reproduction

techniques, importance of disease activity assessment, fetal monitoring, risk of gynecological malignancies associated with exposure to immunosuppressive drugs, and human papillomavirus immunization.

In this special issue, original research articles will focus on the most recent advances on the influence of autoantibodies on fertilization, the impact of SLE, APS, and endometriosis on pregnancy.

The anticentromere antibody (ACA) could impair oocyte maturation potential and early embryonic development, but the mechanisms of this pathology are still unknown. Thus, Y. Ying et al. conducted a preliminary exploration to determine if ACA could penetrate into living mouse embryos and impair their developmental potential. An immunofluorescence assay was performed to determine penetration of polyclonal anticentromere antibodies in the embryos. They show that anti-CENP-A antibody binds to the nucleus of exposed embryos which results in significant growth impairment. These novel results based on *in vitro* observations provide a direction for studies considering mechanisms of ACA influence on embryos and confirmation of these findings in *in vivo* studies.

Antinuclear antibodies (ANAs), including anti-dsDNA antibodies, could be associated with infertility, a decline of oocyte quality and impairment of embryo development, recurrent spontaneous abortion, and *in vitro* fertilization (IVF) failure. Research by J. Fan and colleagues explored whether the anti-dsDNA antibody could adversely affect reproductive outcomes. A total of 259 women receiving *in vitro* fertilization-embryo transfer cycle (IVF) were enrolled in this study including women positive for ANA and anti-dsDNA, positive for ANA and negative anti-dsDNA, and negative for ANA and anti-dsDNA. They compared the number of retrieved oocytes, available embryos and high-quality embryos, rates of implantation, clinical pregnancy and abortion among three groups in fresh embryo transfer, and frozen-thawed embryo transfer cycles, respectively. The authors found that all pregnant patients suffered from abortion in the ANA+/anti-dsDNA+ group which suggests the need of new approaches to understand the role of autoantibodies, especially the involvement of anti-dsDNA, in reproductive outcomes.

Systemic lupus erythematosus is a chronic, multisystem autoimmune disease mostly affecting females of childbearing age and provides challenges in the prepregnancy, antenatal, intrapartum, and postpartum periods for patients and the medics providing care. S. J. Kroese and coauthors had the opportunity to pool data from the rheumatology and obstetric departments of two tertiary centers during a 16-year period. They investigated SLE disease activity before, during, and after pregnancy. Moreover, they examined maternal and perinatal complications in this population, antiphospholipid antibody status, pregnancy complications, and the total number of live births from SLE patients. The authors concluded that in the examined SLE population, the incidence of maternal and perinatal complications is high compared to the reported rates in the general population, irrespective of antiphospholipid antibody status, and despite low disease activity before, during, and after pregnancy. These results obtained during long period observational study could be of additional value in the counseling of SLE patients.

Therapy of rheumatic disorders with antimalarials, especially hydroxychloroquine (HCQ), is well confirmed. The research paper authored by S. J. Kroese et al. investigates if HCQ treatment during pregnancy in women with SLE is associated with improved pregnancy outcomes. The analyzed data was obtained at the University Medical Center Utrecht between 2000 and 2015, and sixty-three SLE patients were included. Hydroxychloroquine use was associated with longer pregnancy duration in the vulnerable preterm birth population, underscoring a beneficial effect of HCQ use during pregnancy. However, the authors state that retrospective and observational character of their study could be its limitation.

Antiphospholipid syndrome is a leading acquired cause of thrombosis and pregnancy loss. High mobility group box 1 (HMGB1) is nuclear protein which organizes DNA and regulates transcription. Furthermore, HMGB1 could play a pivotal role in chronic inflammation and autoimmune diseases. In the manuscript "Elevated Serum Level of HMGB1 in Patients with the Antiphospholipid Syndrome" by V. Manganelli et al., the authors described that HMGB1/sRAGE may play a pathogenic role in recurrent abortion in primary and secondary APS. They analyzed sera from 30 patients with antiphospholipid syndrome (11 primary and 19 secondary APS), 35 subjects with pregnancy morbidity, and 30 healthy females for HMGB1 and its putative receptor RAGE (sRAGE) as well as for TNF. The authors suggest that monitoring HMGB1/sRAGE together with other prognostic parameters represents a useful tool to evaluate risk stratification in prevention of adverse pregnancy outcomes and further studies are warranted.

Endometriosis (EMS) is a common gynecologic disease which could be associated with infertility in women. The role of menstruation back flow planting and defects in the immune system in the pathogenesis of EMS are well established and widely accepted. Currently, we know that endometriosis shares similarities with autoimmune disorders, which include elevated levels of cytokines, decreased apoptosis, autophagy, and cell-mediated pathologies. In their original research paper, M. Gogacz et al. demonstrate that in patients with EMS, peritoneal fluid (PF) macrophages express CD95+ at a significantly higher level than in a nonendometriotic group. Moreover, the concentration of the soluble form of Fas in PF of patients with moderate and severe endometriosis was significantly higher when compared to controls. The authors suggest that in the peritoneal cavity of patients with EMS, a local immune response could not be effective in elimination of misplaced endometrial cells because of increased apoptosis of immune cells like macrophages.

In summary, this special issue covers many important aspects of bidirectional associations between autoimmunity and reproductive health and pregnancy. We hope that this special issue can provide valuable information to researchers as well as clinicians and lead not only to enhancement of knowledge but also to serve for developing better care of pregnant patients with autoimmune diseases.

## Acknowledgments

We would like to thank all the authors for the quality of their submissions that will provide useful insights into the current state of the field. We would also like to express our great appreciation to all the special issue reviewers and editors, whose efforts substantially contributed to the improvement of the overall quality of this thematic issue.

*Jacek Tabarkiewicz*
*Senthamil R. Selvan*
*Nathalie Cools*

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 4570715, 7 pages
https://doi.org/10.1155/2017/4570715

*Research Article*

# Elevated Serum Level of HMGB1 in Patients with the Antiphospholipid Syndrome

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Received 26 May 2017; Revised 7 November 2017; Accepted 14 November 2017; Published 19 December 2017

Academic Editor: Jacek Tabarkiewicz



Pregnancy problems are common in patients with rheumatic disease; indeed, autoimmune disorders and autoantibodies can affect pregnancy progress and lead to maternal complications. Recent studies have highlighted a close association between HMGB1, chronic inflammation, and autoimmune diseases. Thus, in this investigation, we analyzed serum levels of HMGB1, an alarmin which plays a pivotal role in inducing and enhancing immune cell function. Sera from 30 patients with antiphospholipid syndrome (11 primary and 19 secondary APS), 35 subjects with pregnancy morbidity, and 30 healthy women were analysed for HMGB1 and its putative receptor RAGE (sRAGE) by Western blot and for TNF-$\alpha$ by ELISA. Results revealed that APS patients showed significantly increased serum levels of HMGB1, sRAGE, and the proinflammatory cytokine TNF-$\alpha$, as compared to healthy women. However, also, the pregnancy morbidity subjects showed significantly increased levels of HMGB1 and sRAGE as well as TNF-$\alpha$ compared to healthy women. Our findings suggest that in subjects with pregnancy morbidity, including obstetric APS, elevated levels of HMGB1/sRAGE may represent an alarm signal, indicating an increase of proinflammatory triggers. Further studies are needed to evaluate the role of HMGB1/sRAGE as a possible tool to evaluate the risk stratification of adverse pregnancy outcomes.

## 1. Introduction

Inflammatory processes are implicated in every step of fertility, including early pregnancy (implantation and decidualization) [1]. However, recent evidence revealed that inflammatory triggers can lead to adverse pregnancy outcomes, such as preterm birth [2].

Understanding the mechanisms by which inflammation is untimely triggered in the uterus is fundamental to developing effective therapeutics to improve fertility and decrease poor obstetrical outcomes.

Recent studies have highlighted a close association between *high mobility group box 1 (*HMGB1), chronic inflammation, and autoimmune diseases [3, 4]. HMGB1 is a 30 kDa nuclear protein which organizes DNA and regulates transcription; it has been shown to play an important role in helping the recombination activating gene (RAG) endonuclease to form a complex during VDJ recombination. In addition, it was also found in cytosol, mitochondria, and cell plasma membrane, where it can be released to the extracellular milieu [5, 6]. In particular, during inflammation, HMGB1 may be secreted by immune cells, such as macrophages, monocytes, and dendritic cells. This molecule shows all the typical features of alarmins and plays a pivotal role in inducing and enhancing immune cell function, as well as in tissue injury and repair [7, 8]. HMGB1 can interact with Toll-like receptors (TLRs) and activates cells through multiple surface receptors, including Toll-like receptor 2 (TLR2), Toll-like

Table 1: Clinical characteristics of APS patients.

| Characteristics, $n$ (%) | APS ($n = 30$) | SAPS ($n = 19$) | PAPS ($n = 11$) |
|---|---|---|---|
| Vascular thrombosis | 28 (93.3) | 19 (100) | 9 (81.8) |
| Venous thrombosis | 18 (60) | 13 (68.4) | 5 (45.4) |
| Arterial thrombosis | 13 (43.3) | 8 (42.1) | 5 (45.4) |
| Recurrent thrombosis | 12 (40) | 9 (47.4) | 3 (27.3) |
| Pregnancy morbidity | 9 (30) | 5 (26.3) | 4 (36.4) |
| Normal fetus deaths | 2 (6.7) | 1 (5.26) | 1 (9.1) |
| Premature births | 0 | 0 | 0 |
| Spontaneous abortions | 8 (26.7) | 5 (26.3) | 3 (27.3) |
| Vascular thrombosis and pregnancy morbidity | 7 (23.3) | 5(26.3) | 2 (18.2) |
| Noncriteria APS features | 24 (80) | 16 (84.2) | 24 (80) |
| Livedo reticularis | 12 (40) | 8 (42.1) | 4 (36.4) |
| Thrombocytopenia | 7 (23.3) | 4 (21) | 3 (27.3) |
| Migraine | 7 (23.3) | 6 (31.6) | 1 (9.1) |
| Seizures | 4 (13.3) | 3 (15.8) | 1 (9.1) |

APS: antiphospholipid syndrome; PAPS: primary APS; SAPS: secondary APS.

receptor 4 (TLR4), the receptor for advanced glycation end-products (RAGE), leading to an upregulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), with production and release of cytokines, stimulating reactive oxygen species (ROS) release [9–12].

Recently, two isoforms of HMGB1 (28 and 30 kDa) have been characterized in human placenta and were shown to be highly expressed in preeclampsia [13]. Further studies revealed that the mRNA levels of HMGB1, RAGE, and NF-kB were increased in severe preeclamptic placentas. Moreover, increased level of HMGB1 was detected in sera of patients suffering from severe preeclampsia [14]. Other alarmins, including S100 calcium-binding protein A8 (S100A8), were also found to be elevated in early pregnancy loss [15]. However, the specific contribution of alarmins, mainly HMGB1, in these conditions is still under debate. Circulating increased HMGB1 levels have been shown during severe sepsis [16], pneumonia [17], systemic lupus erythematosus (SLE) [18–20], and in the synovial fluid of patients with rheumatoid arthritis [21]. In particular, in SLE patients, serum HMGB1 levels correlated with systemic lupus erythematosus disease activity index (SLEDAI), proteinuria, and anti-ds-DNA antibodies, showing a negative correlation with complement C3 [19, 22].

On the other hand, several autoimmune phenomena have been reported in a wide spectrum of obstetric complications, ranging from eclampsia to recurrent miscarriages [23]. In particular, in the pathogenesis of recurrent spontaneous abortion (RSA), immunological factors have been involved, such as decidual cells, complement system, cytokines, and genes of the histocompatibility complex that can determine the success or the failure of a pregnancy [24, 25]. A deeper insight into apparently unexplained recurrent spontaneous abortion shows increasing evidences supporting autoimmune mechanisms. The best-characterised pathogenic autoantibodies are antiphospholipid antibodies (aPL), and also other autoantibodies, such as anti-Ro/SSA and anti-La/SSB, have been found to be associated with an increased rate of abortion, poor pregnancy outcome, and several other obstetric manifestations [26, 27]. However, it is possible to find patients with RSA who persistently test negative for autoantibodies, including aPL [28–30].

Thus, in this investigation, we decided to analyze serum levels of HMGB1, which may be considered an endogenous sterile driver of inflammation and/or autoimmune response, in order to verify whether high levels of this molecule may represent an alarm signal for pregnancy morbidity.

## 2. Materials and Methods

*2.1. Patients.* The study included 30 consecutive patients, attending the Lupus Clinic, Rheumatology Unit of the Sapienza University of Rome, diagnosed as affected by APS according to the Sydney Classification Criteria [31]; they included both primary APS ($N = 11$) and APS associated with SLE ($N = 19$).

In addition, we enrolled as control group, 35 subjects affected by pregnancy morbidity tested persistently negative (at least 2 times 12 weeks apart) for conventional anticardiolipin (aCL) antibodies, anti-$\beta_2$-glycoprotein I (a$\beta_2$-GPI) antibodies, and lupus anticoagulant (LA) tests [29, 31].

Finally, 30 healthy women of fertile age (normal blood donors) were studied as controls. This study was approved by the local ethic committees and participants gave written informed consent. Sera were collected at several times and stored at −20°C until use.

*2.2. ELISA for aCL and a$\beta_2$-GPI Antibodies.* aCL and a$\beta_2$-GPI antibodies were tested in all the patients' and healthy donors' sera by enzyme-linked immunosorbent assay (ELISA) kits obtained from Inova Diagnostic Inc. (San

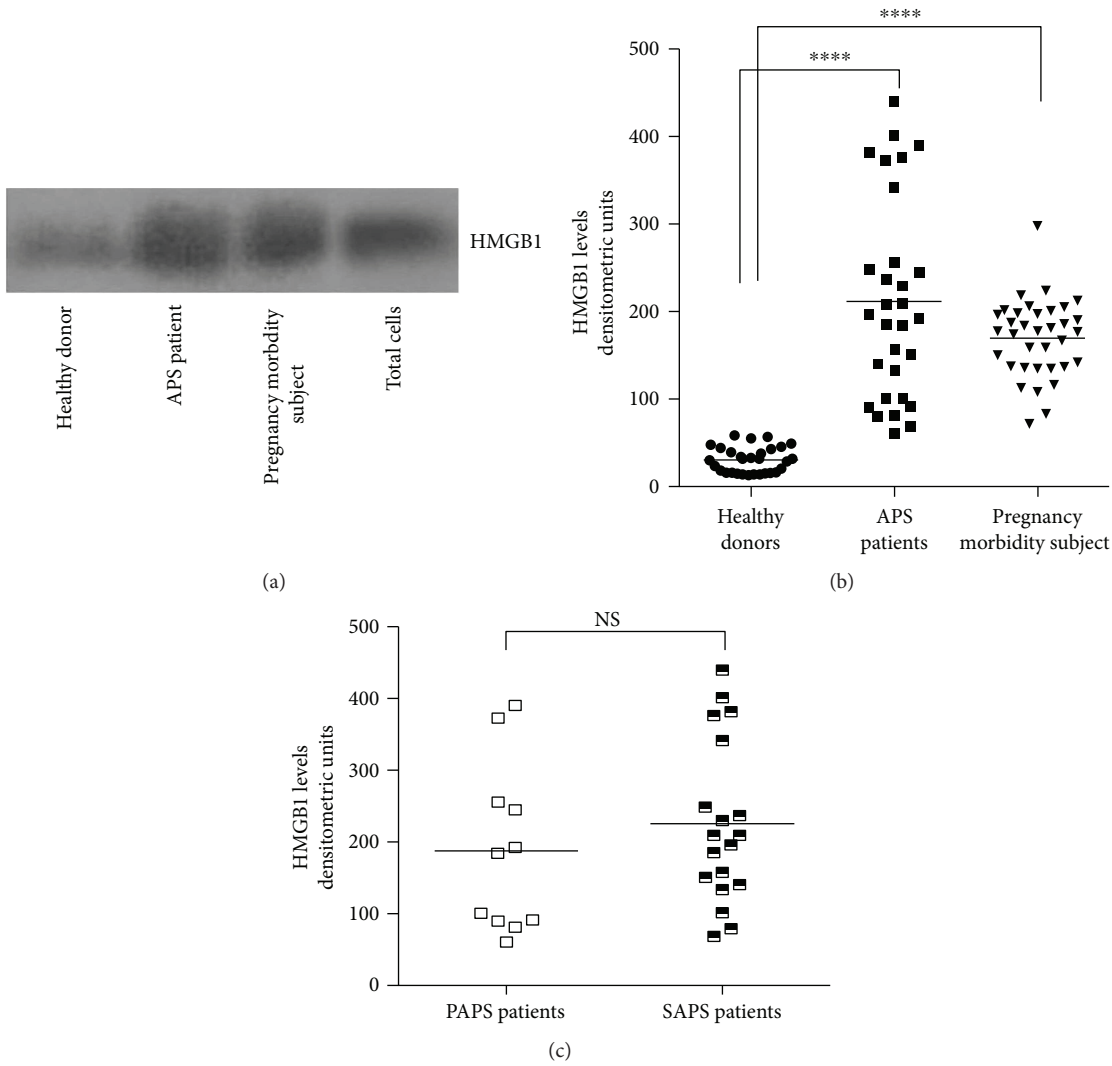


Figure 1: (a) Western blot analysis of HMGB1 expression in the serum of APS patients, subjects with pregnancy morbidity, and healthy donors. A lysate of Jurkat T cells (total cells) was analysed as a positive control. A representative blot for each group is shown. (b) Scatter plot analysis of HMGB1 expression levels in APS patients ($n = 30$), subjects with pregnancy morbidity ($n = 35$), and in healthy donors ($n = 30$). The data are presented as densitometric units. The horizontal bars indicate the mean. Serum HMGB1 levels from both APS patients and subjects with pregnancy morbidity were compared to healthy donors. $^{****}p < 0.0001$. (c) Scatter plot analysis of HMGB1 expression levels in primary APS (PAPS) ($n = 11$) and secondary APS (SAPS) patients ($n = 19$). NS: not significant.

Diego, CA, USA). ELISA was performed according to the manufacturer's instructions.

*2.3. LA Assay.* LA was studied in two coagulation systems, a dilute sensitized activated partial thromboplastin time (aPTT) and a dilute Russell's viper venom time (dRVVT), followed by a confirmation test using reagents and instrumentation by Hemoliance Instrumentation Laboratory, Lexington, MA, USA.

*2.4. Western Blot.* Sera (3 $\mu$l) from subjects with pregnancy morbidity, patients with APS, and healthy donors were diluted with 72 $\mu$l radioimmunoprecipitation assay (RIPA) buffer and heated at 95°C for 5 min in sodium dodecyl sulphate- (SDS-) loading buffer [32]. For immunodetection, the proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) transfer membranes (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked at room temperature for 1 h with Tris-buffered saline that contains 25 mM Tris-HCl, 150 mM NaCl, pH 7.4, and 0.05% Tween-20 (TBS-T) with 3% bovine serum albumin (BSA). The membranes were incubated with primary antibodies: anti-HMGB1 polyclonal antibody (1 : 1000; Abcam, Cambridge UK) or anti-RAGE monoclonal antibody (1 : 1000; Millipore, Billerica, MA, USA). The primary antibody was applied for 2 h at room temperature, followed by four 15 min washes with TBS-T. The secondary antibody was horseradish peroxidase-conjugated anti-rabbit (1 : 10,000; Sigma-Aldrich, Milan,

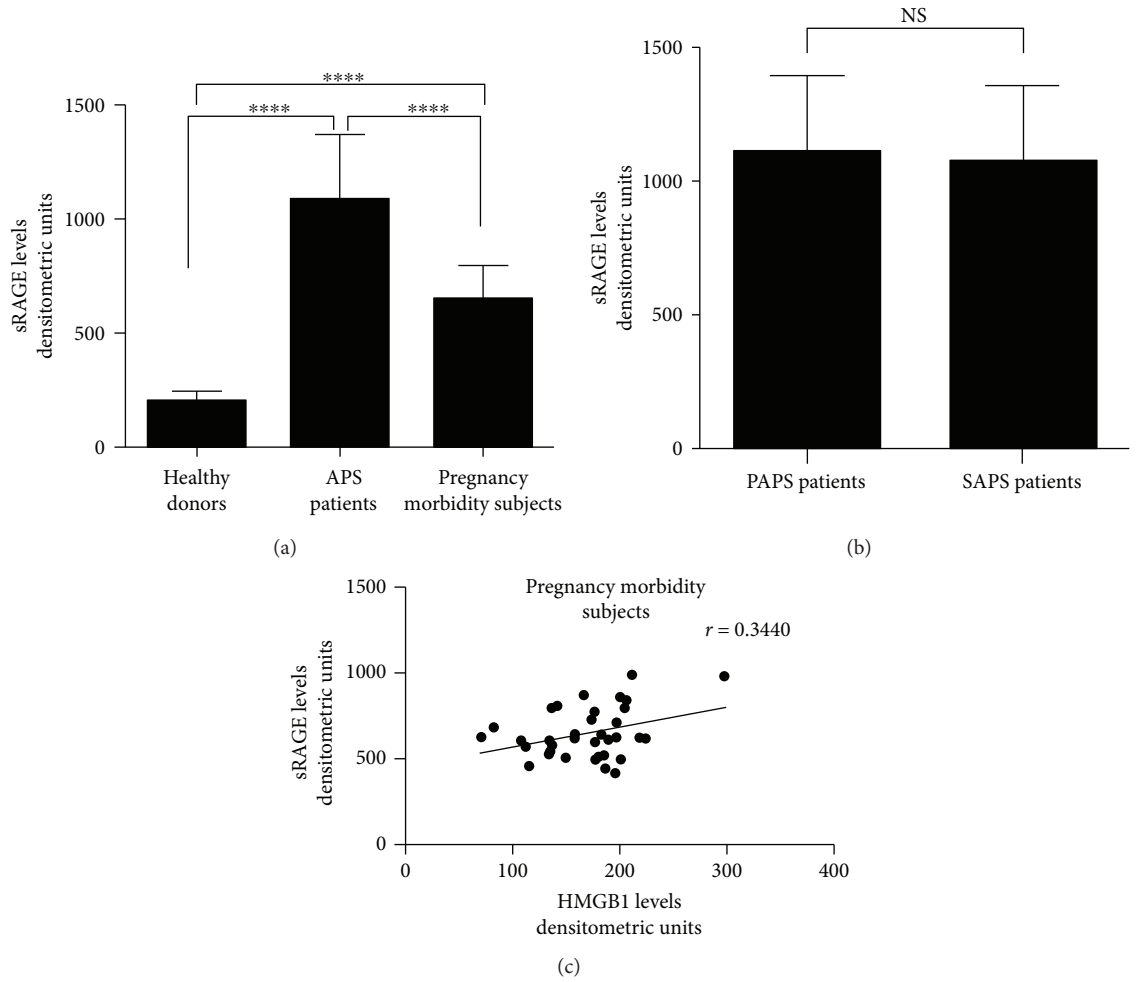


Figure 2: (a) Densitometric analysis of sRAGE levels detected by Western blot in APS patients ($n = 30$), subjects with pregnancy morbidity ($n = 35$), and healthy donors ($n = 30$). Bars represent the mean values; error bars indicate SD. $^{****}p < 0.0001$. (b) sRAGE levels were detected by Western blot in PAPS ($n = 11$) and SAPS patients ($n = 19$). NS: not significant. (c) Scatter plot analysis of serum sRAGE levels versus serum HMGB1 levels in subjects with pregnancy morbidity. Statistically significant correlation was found between sRAGE and serum HMGB1 levels ($r = 0.3440$, $p = 0.0430$).

Italy) or anti-mouse (1 : 5000; Amersham Biosciences) IgG, which was incubated for 1 h at room temperature. After washing, proteins were detected using ECL reagents (Amersham Biosciences). A standard sample was prepared by adding SDS buffer to human Jurkat cells and was included in each blot as an internal control. Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

*2.5. Determination of Serum TNF-α Levels.* Human tumour necrosis factor alpha (TNF-α) was tested in all the patients' and healthy donors' sera by the ELISA, using QuantiGlo Human TNF-α kit (R&D Systems Inc., Minneapolis, MN, USA). The minimal detectable level was 0.35 pg/mL.

*2.6. Statistical Analysis.* All the statistical procedures were performed by GraphPad Prism Software Inc. (San Diego, CA, USA). Normally distributed variables were summarized using the mean ± standard deviation (SD), and nonnormally distributed variables were by the median and range. Differences between numerical variables were tested with the Wilcoxon test. $p$ values less than 0.05 were considered significant. Pearson's correlation coefficient ($r$) was used to assess correlation between sRAGE levels and HMGB1 levels.

## 3. Results

*3.1. Characteristics of Patients.* All 30 APS patients enrolled in this study were Caucasian females with a mean age of 34.3 years (range 17–49), and a mean disease duration of 5.5 years (range 0.1–16). The clinical characteristics of APS patients are reported in Table 1.

Subjects with pregnancy morbidity ($N = 35$) showed a mean age of 36.7 years (range 28–43); none of these subjects experienced thrombotic events. Among these subjects, 11 (27.5%) experienced fetal deaths, 1 (2.86%) premature births, and 25 (62.5%) three or more spontaneous abortions. In this group, two subjects had both spontaneous abortion and normal fetus deaths.

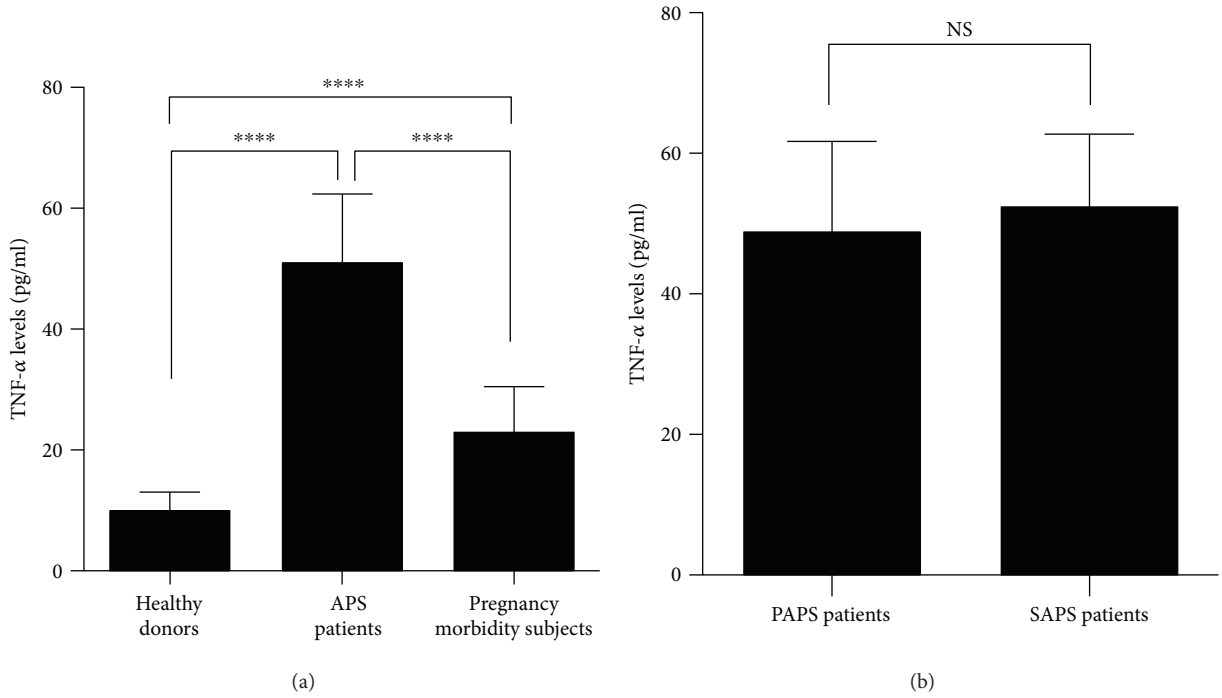


Figure 3: (a) Detection of serum TNF-$\alpha$ levels by ELISA. Serum TNF-$\alpha$ levels from APS patients, subjects with pregnancy morbidity, and healthy donors were compared. $^{****}p < 0.0001$. (b) Serum TNF-$\alpha$ levels were analyzed in PAPS ($n = 11$) and SAPS patients ($n = 19$) by ELISA. NS: not significant.

None of the healthy women of fertile age experienced arterial or venous thrombosis or pregnancy morbidity.

*3.2. Analysis of Circulating HMGB1 in APS Patients and Subjects with Pregnancy Morbidity.* Since HMGB1 is an alarmin, whose circulating levels may be elevated during chronic inflammation, autoimmune diseases, or preeclampsia, in this investigation, we preliminarily tested HMGB1 expression by Western blot in sera from patients with APS patients, compared with women with pregnancy morbidity and healthy blood donors (Figure 1(a)). The results showed that virtually all the APS patients, either primary (PAPS) or secondary (SAPS), as well as the subjects with pregnancy morbidity showed increased serum levels of HMGB1, as compared to healthy women, as revealed by densitometric analysis (Figure 1(b)). Thus, HMGB1 serum levels of both APS patients and pregnancy morbidity subjects were significantly higher than healthy controls ($p < 0.0001$). Furthermore, no significant differences of HMGB1 levels between primary and secondary APS were found (Figure 1(c)).

Among APS patients, HMGB1 serum levels were not different in subjects with thrombotic events and in those with pregnancy morbidity; both of them presented serum HMGB1 levels significantly increased in comparison to healthy controls ($p < 0.0001$).

*3.3. Analysis of sRAGE Levels in APS Patients and Subjects with Pregnancy Morbidity.* Since RAGE has been identified as the specific receptor for extracellular HMGB1, we further analyzed soluble RAGE (sRAGE) in sera of APS patients, subjects with pregnancy morbidity, and healthy blood donors. The results showed that both APS patients and subjects with pregnancy morbidity showed significantly increased levels of sRAGE as compared to healthy women ($p < 0.0001$, Figure 2(a)). No significant differences of serum sRAGE levels between primary and secondary APS were detected (Figure 2(b)).

A significant correlation was found between HMGB1 and sRAGE levels in the subjects with pregnancy morbidity ($r = 0.3440$, Figure 2(c)).

*3.4. Analysis of TNF-α Levels in APS Patients and Subjects with Pregnancy Morbidity.* We then decided to test TNF-$\alpha$ levels, considering this molecule as a possible proinflammatory cytokine in APS patients. The highest values were detected in sera of APS patients. Statistical analysis revealed that TNF-$\alpha$ levels of APS patients were significantly higher as compared to both pregnancy morbidity subjects and healthy donors ($p < 0.0001$, Figure 3). However, also the pregnancy morbidity subjects showed significantly increased levels of TNF-$\alpha$ as compared to healthy women ($p < 0.0001$) (Figure 3(a)). We did not find significant differences in TNF-$\alpha$ levels between primary and secondary APS (Figure 3(b)).

## 4. Discussion

In this study, we show elevated serum levels of the alarmin HMGB1 in patients with APS and in subjects with pregnancy morbidity. As a consequence, we also found in both groups

significantly increased levels of sRAGE, the putative receptor for extracellular HMGB1.

These findings are not surprising, since it is well known that HMGB1 may play a role in inducing and enhancing innate immunity, and, secondly, inflammatory and autoimmune phenomena may be involved in a wide spectrum of obstetric complications [7, 13, 14, 33].

In particular, we show for the first time the increased levels of HMGB1 in APS patients. Interestingly, we observed this phenomenon not only in secondary APS (elevated levels of HMGB1 during SLE have already been reported) [19, 34] but also in primary APS. During pregnancy, proinflammatory stimuli have been associated with higher risk of adverse pregnancy outcomes, such as preterm birth [2, 35]. In particular, HMGB1, once accumulated in the extracellular milieu, is able to convey danger signals by triggering inflammatory patterns with extracellular signal-regulated kinases (ERKs), p38, and NF-kB activation via several cell surface receptors, including TLR2, TLR4, CD24, and, mainly, RAGE [12, 16, 36].

From a technical point of view, we decided to test HMGB1 by Western blot, instead of ELISA, to avoid the possibility that serum/plasma components able to bind to HMGB1 may interfere with its detection [32].

To confirm that also in our patients HMGB1/RAGE system represents an endogenous "driver" of inflammation, we tested the levels of TNF-$\alpha$, the most typical proinflammatory cytokine, and observed that APS patients showed significantly increased levels of TNF-$\alpha$, as compared to control subjects. Thus, we can conclude that also in APS patients the increase of serum level of HMGB1 is accompanied by high levels of TNF-$\alpha$. It is in agreement with similar observation in SLE patients, where elevated plasma level of HMGB1 is associated with disease activity and combined alterations with TNF-$\alpha$ [20].

As several studies have shown elevated levels of HMGB1 in pregnancies at high risk of developing complications associated with placental dysfunction such as growth restriction or preterm labor [37] and preeclampsia [38] for the first time, we also aimed to evaluate serum levels of this alarmin in subjects with pregnancy morbidity.

Our findings suggest that in subjects with pregnancy morbidity, including obstetric APS, elevated levels of HMGB1/sRAGE may represent an alarm signal, indicating an increase of proinflammatory triggers. However, this finding cannot be considered highly specific, since we ourselves reported that several events, including surgical/anesthesia trauma, induce an early intracellular upregulation of HMGB1 in monocytes, with consequent release of the alarmin in serum [39].

In conclusion, HMGB1/sRAGE may play a role in monitoring of pregnancy morbidity risk. Additional studies are needed to demonstrate that monitoring HMGB1/sRAGE together with other prognostic parameters may represent a useful tool to evaluate the risk stratification, in order to prevent adverse pregnancy outcomes.

## Conflicts of Interest

The authors declare that they have no competing interests.

## Authors' Contributions

Valeria Manganelli and Antonella Capozzi contributed equally to this work. Agostina Longo and Roberta Misasi are the senior authors.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 2810202, 5 pages
https://doi.org/10.1155/2017/2810202

*Research Article*

# Hydroxychloroquine Use in Lupus Patients during Pregnancy Is Associated with Longer Pregnancy Duration in Preterm Births

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Received 7 May 2017; Accepted 26 September 2017; Published 17 December 2017

Academic Editor: Jacek Tabarkiewicz



*Objective*. To investigate the effect of hydroxychloroquine (HCQ) in pregnant women with systemic lupus erythematosus (SLE). *Methods*. In SLE pregnancies of a single Dutch center (2000–2015), lupus activity and flares before and during pregnancy and postpartum were assessed using the SLE Disease Activity Index (SLEDAI)/SLEPDAI (SLEDAI adjusted for pregnancy). The association between HCQ use and pregnancy outcomes (early spontaneous abortion, fetal death, and preterm and term live birth) was analyzed using generalized estimating equations (GEE) accounting for the occurrence of multiple pregnancies per patient. Analyses were adjusted for antiphospholipid antibody (aPL) status. *Results*. 110 pregnancies (63 mostly Caucasian patients) were included, of which, in 30, HCQ was used; overall occurrence of flares was low (non-HCQ group: 5 mild (6.4%) and 2 severe (2.6%); HCQ group: 2 mild (6.7%) and no severe flares). The HCQ group showed a trend towards lower dosage of prednisone (OR 0.2 (95% CI 0.0–1.4); $p = 0.10$). Pregnancy outcomes were comparable between groups. Among preterm live births, pregnancy duration was significantly longer in HCQ users (2.4 weeks (95% CI 1.0–3.8; $p \leq 0.001$)). *Conclusion*. HCQ use was associated with longer pregnancy duration in the vulnerable preterm birth population, underscoring the beneficial effect of HCQ use during pregnancy.

## 1. Introduction

Pregnancy constitutes a challenge in patients with systemic lupus erythematosus (SLE). Apart from disease flares during pregnancy, SLE patients have an increased risk of intrauterine growth restriction (OR 2.6), (pre)eclampsia (OR 3.0), and preterm birth (OR 2.4) compared to the healthy population [1]. In SLE patients, the antimalarial drug hydroxychloroquine (HCQ) is not only used for the treatment of skin lesions and arthritis but also for a more general goal, namely, prevention of cardiovascular disease and flares. The use of HCQ during pregnancy has long been debated, but nowadays, consensus is reached that it is safe and it is frequently prescribed during pregnancy to diminish flares [2, 3]. However, reports on the detailed effects of HCQ on pregnancy outcomes are scarce and mostly focus on teratogenicity. Recently, a French retrospective study of 118 pregnancies in SLE patients who delivered after 22-week gestation or longer found less preterm birth and intrauterine growth restriction in women who used HCQ during pregnancy compared to those who did not use HCQ in the six months prior to or during pregnancy [4]. Our study is the first to investigate multiple pregnancies in SLE women in a tertiary center in order to provide insight into the effects

of HCQ use on pregnancy outcomes in a (homogenous) Dutch SLE population.

## 2. Methods

Data of all pregnancies between 2000 and 2015 in women with SLE seen at the University Medical Center (UMC) Utrecht were retrieved from patient medical files, from an intern SLE registry, and from an in-house obstetric registry. SLE was classified according to the 1997 American College of Rheumatology (ACR) criteria [5]. Disease manifestations and duration at the start of pregnancy as well as treatment during pregnancy were recorded. Disease activity was assessed using the SELENA-SLEDAI score or SLEPDAI score where appropriate (SLEDAI adjusted for pregnancy) six months before and during pregnancy as well as six weeks postpartum [6, 7]. Flares were classified according to the SLE(P)DAI scores as mild/moderate or severe [6, 7]. Pregnancy outcomes were categorized according to pregnancy duration as early spontaneous abortion (<10 weeks of gestation), fetal death (>10 weeks of gestation), preterm live birth (PTLB; live birth < 37 weeks), and term live birth (TLB; live birth ≥ 37 weeks). Terminated pregnancies due to social reasons were not included in this study. Preeclampsia was defined as hypertension (>140 mmHg systolic or >90 mmHg diastolic with previous normal tension) combined with proteinuria (>0.3 g/24 h) [8]. HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome was defined following the Tennessee criteria as high LDH (>600 U/l) combined with low platelets ($<100 \times 10^9$/l) and elevated liver enzymes (ASAT > 70) [9]. Incomplete HELLP (iHELLP) fulfilled one or two of the required three items of the HELLP syndrome. Small for gestational age (SGA) was defined as birth weight below the 10th percentile (<p10). The Ethics Committee at the UMC Utrecht provided approval for the SLE registry, and all patients gave written informed consent.

Data were described using mean and standard deviation (SD) or median and interquartile range (IQR) where appropriate. As several of the patients had had multiple pregnancies, the association between HCQ use and pregnancy outcomes as well as pregnancy duration was analyzed using generalized estimating equations (GEE), correcting for patient dependency of observations, using an exchangeable correlation structure. GEE corrects for within-subject correlations in repeated measurements by the use of a correlation structure for the repeated measurements and corrects for standard errors of the regression coefficient [10, 11].

All analyses were adjusted for antiphospholipid antibody (aPL) status (based on the presence or absence of lupus anticoagulant and/or IgG or IgM class anticardiolipin antibodies and/or IgG or IgM anti-$\beta$2-glycoprotein antibodies) except for early spontaneous abortion, for which aPL occurrence was too infrequent for analysis. For the outcomes pregnancy duration, (pre)eclampsia, and (i)HELLP, only pregnancies with duration > 10 weeks of gestation were included ($n = 89$). A two-sided $p$ value < 0.05 was considered statistically significant for all analyses. Statistical analysis was performed using IBM SPSS Statistics version 21.

## 3. Results

Sixty-three SLE patients were included. Their baseline characteristics are presented in Table 1. The percentage of patients fulfilling each ACR criterion cumulatively from the start of each first recorded pregnancy is shown in Figure 1. Neurology was the only ACR criterion that was found significantly more often in the HCQ group ($p < 0.05$); however, absolute numbers are small ($N = 2$ (HCQ) versus $N = 0$ (non-HCQ)).

Of the total group of 63 SLE patients, 110 pregnancies were included. Of the 63 patients, 14 used HCQ in 30 pregnancies. The dose of HCQ used in these pregnancies was 200 mg/day (16 pregnancies) or 400 mg/day (14 pregnancies). Ninety-six of 110 pregnancies (87%) were planned and beforehand medically approved by a rheumatologist or gynecologist at counselling consultations.

Pregnancy outcomes according to HCQ use are shown in Table 2. Hypertensive disorder of pregnancy occurred in 17% of pregnancies, and over 60% of pregnancies ended in full-term birth. Fourteen of eighteen preterm births were iatrogenically induced preterm deliveries due to either maternal ($n = 7$), fetal ($n = 6$), or combined ($n = 1$) indications. Indications included a.o. solutio placentae, severe preeclampsia, and placental insufficiency. There were no statistically significant differences in the occurrence of preterm birth between both groups; however, a substantial, although not significantly proportional, part of patients showed lower occurrence within the HCQ group (6.7% with HCQ use versus 20.0% without HCQ, OR 0.5 (95% CI 0.1–2.4); $p = 0.37$). Among the PTLB, pregnancy duration was significantly longer (2.4 weeks (95% CI 1.0–3.8), $p \leq 0.001$) for pregnancies during which HCQ was used than for those during which no HCQ was used. A prolongation of pregnancy was not observed in pregnancies which ended in term birth. The association between HCQ use and pregnancy duration in PTLB was not confounded by the use of prednisone or azathioprine. However, in HCQ users who smoked, a known risk factor for preterm birth, pregnancy duration was only 1.9 weeks longer (95% CI 0.6–3.2; $p = 0.01$) instead of 2.4 weeks. The rate of SGA children did not statistically significantly differ between the non-HCQ and HCQ groups (12.5 versus 16.7%, OR 2.2 (95% CI 0.6–7.5); $p = 0.22$). One case of neonatal lupus occurred: a congenital heart block in the non-HCQ group. Congenital anomalies included one case of, respectively, hip dysplasia, Loeys-Dietz syndrome, and anal atresia, all in the non-HCQ group.

The median SLE(P)DAI score was 2 (IQR 0–4) six months before conception, for each trimester and for the 6-week postpartum period, irrespective of HCQ use. Six months before conception, there were no severe flares and two mild flares in both groups (6.7% (HCQ) versus 2.5% (non-HCQ)). Mild flare occurrence was 0/2/3/3 in the non-HCQ group and 1/0/1/3 in the HCQ group per trimester and postpartum. Two severe flares occurred, both in the non-HCQ group, in the first and second trimester. Flares

Table 1: Patient characteristics of SLE patients.

| | Total ($N = 63$) | Non-HCQ ($N = 49$) | HCQ ($N = 14$) |
|---|---|---|---|
| Race ($n$ (%)) | | | |
| Caucasian | 56 (89) | 45 (92) | 11 (79) |
| Black | 1 (2) | 1 (2) | 0 (0) |
| Asian | 6 (10) | 3 (6) | 3 (21) |
| Age at the start of the first registered pregnancy (mean (SD)) | 31.0 (4.1) | 30.6 (4.0) | 32.5 (4.3) |
| Disease duration at the start of the first registered pregnancy | 6.0 (3.0–10.0) | 6 (3.5–9.5) | 4.5 (3–10.8) |
| Number of registered pregnancies per patient | 1 (1-2) | 1 (1-2) | 2 (1-2) |
| Antiphospholipid antibodies present | 7 (11) | 6 (13) | 1 (7) |
| LAC ($n$ (%))* | | | |
| Negative | 58 (92) | 44 (90)* | 14 (100) |
| Positive | 4 (7) | 4 (9) | 0 (0) |
| aCL-IgG ($n$ (%)) | | | |
| Negative | 59 (94)* | 45 (92)* | 14 (100) |
| Positive | 3 (54) | 3 (6) | 0 (0) |
| aCL-IgM ($n$ (%)) | | | |
| Negative | 61 (97)* | 48 (98)* | 13 (93) |
| Positive | 1 (2) | 0 (0) | 1 (7) |

SD: standard deviation; IQR: interquartile range. Age and disease duration in years. Data depicted as median (IQR) unless otherwise indicated. *Unknown: $n = 1$. Anti-beta2 glycoprotein IgG/IgM was tested in 31 patients in whom it was all negative.

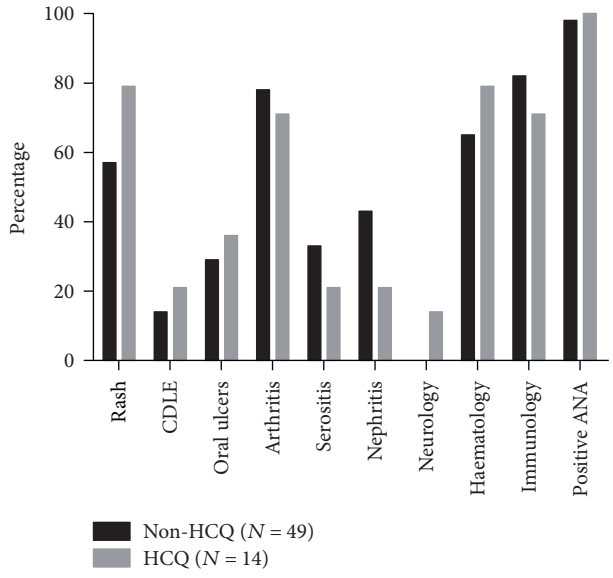


Figure 1: Percentage of patients fulfilling each ACR criterion cumulatively from the start of every first registered pregnancy. Results are separately shown for patients who used HCQ or did not use HCQ during the first registered pregnancy. CDLE: chronic discoid lupus erythematosus; ANA: antinuclear antibodies.

were usually characterized by arthritis (40%), low complement levels (27%), proteinuria (27%), and/or a rise in anti-dsDNA (20%).

In 57% of pregnancies, prednisone was used (Table 2). HCQ use was not associated with prednisone use (53.8% (non-HCQ) versus 60.0% (HCQ), OR 0.9 (95% CI 0.7–1.2); $p = 0.35$). However, in the pregnancies with prednisone therapy, there were distinctively more patients with a higher dosage of prednisone (>7.5 mg) in the non-HCQ group compared to the HCQ group, although this did not reach statistical significance (17.5% (non-HCQ) versus 73.3% (HCQ), OR 0.2 (95% CI 0.0–1.4); $p = 0.13$). The use of azathioprine did not differ between the non-HCQ and HCQ pregnancies.

## 4. Discussion

In this study, we investigated the association between HCQ use and disease activity as well as pregnancy outcomes in the SLE population followed at a single center. Our most important finding was that HCQ was significantly associated with longer pregnancy duration in the subgroup of women with preterm delivery. To our knowledge, this has not been reported in SLE pregnancies yet. Several recent studies have shown that neonates of HCQ-using pregnant SLE women reach a higher gestational age at birth [3, 4, 12]. Although this is in line with our findings, none of these described the effect of HCQ on preterm births.

Since fetal cerebral and respiratory developments are known to improve with the length of pregnancy, especially in preterm births, the gain in pregnancy duration of more than a week in this subgroup is of great clinical relevance [13, 14]. A possible explanation for the longer pregnancy duration in HCQ users is the recently described positive effects of antimalarials on endothelial dysfunction, which might preserve placental function and thus pregnancy [15].

Although we did not find a significantly lower occurrence of preterm birth within the HCQ group as described by Leroux et al. [4] (15.8% versus 44.2% ($p = 0.006$)), a trend towards less preterm births within the HCQ group was observed in our study [12].

Table 2: Maternal and fetal pregnancy outcome according to HCQ treatment.

| | Total ($N = 110$) | Non-HCQ ($N = 80$) | HCQ ($N = 30$) | OR (95% CI)[$$]; $p$ value |
|---|---|---|---|---|
| *Maternal outcome* | | | | |
| Preeclampsia[*] | 13 (11.8) | 9 (11.3) | 2 (6.7) | 1.0 (1.0-1.0); 0.57 |
| Eclampsia[*] | 0 (0) | 0 (0) | 0 (0) | — |
| (i)HELLP[*] | 6 (5.5) | 5 (6.3) | 1 (3.3) | 1.3 (0.1–17.9); 0.84 |
| Prednisone use[†] | 63 (57.3) | 43 (53.8) | 18 (60.0) | 0.9 (0.7–1.2); 0.35 |
| Prednisone < 7.5 mg within prednisone users | 36 (32.7) | 14 (17.5) | 22 (73.3) | 0.2 (0.0–1.4); 0.10 |
| *Fetal outcome* | | | | |
| Early spontaneous abortion (<10 weeks of gestation) | 19 (17.3) | 10 (12.5) | 9 (30.0)[▲] | 1.5 (0.3–9.0); 0.66 |
| Fetal death[‡] (>10 weeks of gestation) | 3 (2.7) | 2 (2.5) | 1 (3.3) | — |
| Preterm live birth | 18 (16.4) | 16 (20.0) | 2 (6.7) | 0.5 (0.1–2.4); 0.37 |
| Of which <34 weeks | 5 (4.5) | 5 (6.3) | 0 (0) | — |
| Term live birth | 70 (63.6) | 52 (65.0) | 18 (60.0) | 0.9 (0.3–2.7); 0.90 |
| Small for gestational age | 15 (13.6) | 10 (12.5) | 5 (16.7) | 2.2 (0.6–7.5); 0.22 |
| | | | | $\beta$ (95% CI)[$$]; $p$ value |
| Duration of pregnancy[*] (median, IQR) | 38.9 (37.1–40.0) | 38.9 (36.4–40.1) | 38.7 (37.7–39.4) | −1 (−3.8 to 1.8); 0.48 |
| Duration of pregnancy in preterm live births[#] (median, IQR) | 35.1 (31.5–36.3) | 34.9 (30.9–35.4) | 36.8 (36.7-...) | 2.4 (1.0–3.8); 0.001 |

Data depicted as numbers (%) unless otherwise indicated. HCQ: hydroxychloroquine; IQR: interquartile range; HELLP: (incomplete) hemolysis, elevated liver enzymes, and low platelet syndrome. [$$]Dependent variable: pregnancy outcome/prednisone use/duration of pregnancy. Predictor variable: HCQ use (ref = non-HCQ). Adjusted for antiphospholipid status, except for early spontaneous abortion. [*]Pregnancies ending < 10 weeks of gestation were excluded ($N = 89/68/21$). [†]Prednisone dose was increased in 4.6% of pregnancies. [▲]Of which, 5 occurred within one woman. [‡]Two were due to elective termination, one because of trisomy 21 with Fallot's tetralogy, and one because of infaust prognosis with severe preeclampsia, both occurring within the non-HCQ group. [#]($N = 18/16/2$) duration of pregnancy in weeks.

We did not find a significantly higher rate of SGA in the non-HCQ group compared to the HCQ group as described in other studies [4, 16]. The higher rate of SGA in non-HCQ users in the French cohort compared to our cohort (26% versus 6%) could be explained by the higher occurrence of SLE flares in the former. Previous studies described a trend towards lower SLE disease activity in pregnancies with HCQ use [2, 17]. In our study, disease activity scores and frequency of flares during pregnancy were not significantly different between both groups. This might be due to the fact that our population comprised predominantly Caucasian women with quiescent lupus at the start of pregnancy. The latter is a consequence of the policy in our center to aim at planning parenthood and extensive prepregnancy counselling. In our patient group, 87 percent of pregnancies were considered safe by the consulting physician in prepregnancy counselling. Quiescent disease for at least six months prior to pregnancy is known to reduce flare rates during pregnancy [18].

In our study, women using HCQ showed a trend towards lower prednisone dose throughout their pregnancies, albeit not statistically significant. In a prospective study of 357 pregnancies, a similar trend towards lower prednisone dose during pregnancy was noted within the group of patients using HCQ [2].

The limitation of our study is its retrospective and observational character. Due to the low number of adverse outcomes, our findings might underestimate the effect of HCQ and might be influenced by a type II error. Although the use of HCQ in SLE pregnancies is nowadays encouraged, a recent prospective study analyzing (adverse) outcomes and predictors thereof in pregnancies of SLE patients shows that only 64.7% used HCQ during pregnancy [19]. No data are provided on the effect of this treatment on prolongation of pregnancy duration in preterm deliveries. To confirm that treatment with HCQ in pregnant patients with SLE is beneficial for pregnancy outcome, a large, prospective, double-blinded randomized clinical trial is needed. However, given the well-established role for HCQ as one of the cornerstones of SLE treatment and the results from a recent meta-analysis that confirmed that HCQ can safely be used during pregnancy [16], such a trial does not seem ethical. Our findings provide further grounds for the continuation or even start of HCQ in SLE patients who want to become pregnant.

## Conflicts of Interest

Professor Dr. J.M. van Laar has received honoraria from Eli Lilly, Pfizer, Roche, and MSD and a research grant from Astra Zeneca.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 3175394, 8 pages
https://doi.org/10.1155/2017/3175394

*Research Article*

# Fas-Related Apoptosis of Peritoneal Fluid Macrophages in Endometriosis Patients: Understanding the Disease

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Received 13 June 2017; Revised 25 August 2017; Accepted 10 October 2017; Published 1 November 2017

Academic Editor: Senthami R. Selvan



Recent studies of the peritoneal cavity environment in endometriosis demonstrate quantitative and qualitative changes in the cells responsible for cell-mediated immunity. Such changes may have led to disturbances in the surveillance, recognition, and destruction of misplaced endometrial cells and might have, in fact, brought about the disease. The aim of the study was to assess CD95 (Fas) expression on (activated) peritoneal fluid (PF) macrophages, as well as to ascertain soluble Fas (sFas) concentration in the PF of endometriosis patients, as compared to the nonendometriotic group. The concentration of leukocytes in the PF, the percentage of cells expressing $CD45^+/CD14^+$, and the percentage of PF macrophages expressing the HLA-DR antigen were significantly higher in patients with stages I and II endometriosis. The percentage of Fas- ($CD95^+$-) expressing macrophages was significantly higher in all stages of the disease, in comparison with controls. Moreover, the concentration of sFas in the PF of patients with moderate and severe endometriosis was significantly higher, as compared to the reference group. The high number of immune cells in PF in early stage endometriosis and their increased susceptibility to apoptosis confirm the role of the impaired peritoneal environment and immune defects in the development and progression of the disease.

## 1. Introduction

Endometriosis is characterized by the presence of endometrial-like tissue outside the uterus [1, 2]. From the last decade of the 19th century, onwards, many attempts have been made to develop a definitive theory for explaining the pathogenesis of the disease, but its origin still remains controversial and poorly understood [3–6]. Still, current theory holds that retrograde menstruation, first described by Sampson, serves as a mechanism for the transportation of endometrial tissue which subsequently implants and proliferates at the ectopic sites. However, while the retrograde flow of blood into the peritoneal cavity is observed in 76–90% of all women, endometriosis develops only in 15% of all women of reproductive age. This suggests the involvement in the pathogenesis of endometriosis of other factors, such as impaired peritoneal environment [7].

It is well recognized that cell-mediated immune response contributes to the elimination of foreign antigens and misplaced autologous cells such as ectopic endometrial cells [8]. This mechanism may, hence, also protect most women against endometriosis. Therefore, alterations in cell-mediated immunity may promote the development of the disease [9]. Furthermore, in the peritoneal cavity, the peripheral blood monocytes (PBM), as well as the peritoneal macrophages (PM), represent the primary line of host defense against endometriosis. These multifunctional immune cells may also control the activity of other cells and play a key role in the pathogenesis of endometriosis [10, 11].

Apoptosis is one of the mechanisms responsible for the hemostasis of the immune system which eliminates activated

and inactivated cells. Several pro- and antiapoptotic factors have been identified [12, 13]. The Fas antigen (APO-1/CD95) is a glycosylated 48 kDa surface protein and is a member of the tumour necrosis factor/nerve growth factor receptor superfamily with proapoptotic properties [14]. Fas is expressed in various human tissues, including immune cells, and in many different tumour cells [15–18]. The triggering of Fas by its ligand (FasL) or by certain anti-Fas agonistic antibodies results in rapid induction of programmed cell death in susceptible Fas-bearing cells.

The expression of Fas cell surface protein is enhanced by the activation of lymphocytes by IFN-$\gamma$ and TNF [19]. An enzymatically exfoliated extracellular domain of Fas receptor (sFas) is present in the body fluids, but its role is not well known yet. Probably, sFas competes with membrane Fas for the binding of the Fas ligand. The malfunction of the Fas system induces immune disorders and promotes the occurrence of autoimmune disease, whereas its exacerbation may cause tissue destruction.

Recent studies of the peritoneal cavity environment in women with endometriosis demonstrate quantitative and qualitative (functional) changes in monocytes/macrophages and natural killer cells, as well as the cytotoxic T and B lymphocytes responsible for cell-mediated immunity. The observed changes may disturb the surveillance, recognition, and destruction of misplaced endometrial cells and, hence, induce impairment of immune cell-mediated response in the peritoneal cavity. This situation possibly leads to endometriosis.

The aim of the study was to assess CD95 (Fas) expression on (activated) peritoneal fluid (PF) macrophages, as well as to ascertain soluble Fas (sFas) concentration in the peritoneal fluid of patients with endometriosis, as compared to the nonendometriotic group.

## 2. Material and Methods

*2.1. Ethics and Informed Consent.* The study was conducted at II Department of Gynecology, Medical University of Lublin (Poland). The protocol was reviewed and approved by the Institutional Review Board of the Medical University of Lublin before the start of the study. All potential participants were provided with a verbal and written informed consent regarding the reasons for the study and listing potential adverse effects. Each woman, by signing the informed consent, acknowledged and accepted the provided information.

*2.2. Patients.* The study group consisted of 26 women of reproductive age with endometriosis as confirmed by histological results. The stage of the disease was assessed according to the revised American Society of Reproductive Medicine classification [20]. Stage I, stage II, and, together, stages III and IV were found in 11, 10, and 5 patients, respectively. The control group consists of 18 women of reproductive age who had undergone surgery due to benign noninflammatory and nonendometrial ovarian cysts. All patients had regular ovulatory cycles varying in duration between 26 and 33 days. Oral contraceptives, hormone-releasing intrauterine devices, or ovulation-inducing drugs were not used for at least 3 months before surgery.

*2.3. Peritoneal Fluid Collection.* Peritoneal fluid (PF) was collected from 44 patients during a laparoscopy that was performed due to infertility or the presence of a benign noninflammatory mass of ovary/ovaries in the midfollicular phase of the cycle (6th–14th day of the cycle). The obtained fluid (3–5 ml) was aspirated under direct vision, from either the Douglas or uterovesical pouch, with a nylon catheter, at the beginning of the surgery, after trocar introduction, and was placed in sterile heparinized tubes (Greiner). Any contamination of the fluid with blood from injured vessels after trocar insertion excluded patients from the analysis.

*2.4. Concentration of Immune Cells in Peritoneal Fluid.* After homogenous dispersion of immune cells, its concentration was counted in Thom's hemocytometer and recorded individually (per patient).

*2.5. Preparation of the Cells.* Mononuclear cells were isolated on density gradient medium (Polymorphprep, Nycomed, Norway) by centrifugation at 600$g$, for 25 minutes, at room temperature. After centrifugation, PF supernatant was aspirated and stored until analysis. The cell pellet from the interphase was then removed, washed twice in phosphate-buffered saline (PBS, Biomed, Lublin) containing 1% bovine serum albumin, and resuspended in polyethylene tubes at $0.5 \times 10^6$ cells each. The prepared cells were incubated with monoclonal antibodies for 30 minutes at 4°C and washed twice afterwards.

*2.6. Phenotyping of Peritoneal Fluid Immune Cells.* Double-colour immunofluorescence studies were performed using combinations of (FITC/PE) monoclonal antibodies—conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin (PE). The following antibody combinations were used:

(i) IgG1 FITC/IgG2a PE (Ortho Diagnostic Systems) (Germany) as negative control

(ii) CD45/14 (Ortho Diagnostic Systems) (Germany)

(iii) CD14/HLA-DR (Ortho Diagnostic Systems) (Germany)

(iv) CD3/19 (Ortho Diagnostic Systems) (Germany)

(v) CD 3/16/56 (Ortho Diagnostic Systems) (Germany)

(vi) CD95 PE (Immunotech)

*2.7. Flow Cytometric Analysis of Expression of CD Antigens.* All samples were measured on a Cytoron Absolute flow cytometer (Ortho Diagnostic System). In this part of the experiment, 10000 cells were collected and analyzed per test for fluorescence. The samples were first stained with propidium iodide, and dead cells that showed staining were excluded from further analysis. In this experiment, the mononuclear cell subpopulation was represented as a percentage of the entire CD45-positive cell population, while PF macrophages were determined by CD14 monoclonal antibody content. To recognize subpopulations of T/B

Table 1: The mean (±SD) concentration of leukocytes, percentage of macrophages, and MFI of CD14 antigen in peritoneal fluid.

| | Endometriotic patients ($n = 26$) | Stage I ($n = 11$) | Stage II ($n = 10$) | Stage III/IV ($n = 5$) | Reference group ($n = 16$) |
|---|---|---|---|---|---|
| Concentration of PF leukocytes (cells/mm$^3$) | $2215 \pm 1030^*$ | $2490 \pm 1116^*$ | $2210 \pm 1099^*$ | $1620 \pm 377$ NS | $1231 \pm 420$ |
| Percentage of PF macrophages CD45$^+$/CD14$^+$ (%) | $83.2 \pm 7.8^*$ | $84.2 \pm 6.5^*$ | $85.4 \pm 5.58^*$ | $67.6 \pm 11.6$ NS | $57.7 \pm 12.4$ |
| MFI of CD14 antigen | $132.8 \pm 32.4$ NS | $119.4 \pm 27.5$ NS | $144.5 \pm 28.2$ NS | $138.9 \pm 45.1$ NS | $135.9 \pm 19.9$ |

*Statistically significant compared to reference group ($p \leq 0.05$). NS: statistically nonsignificant.

lymphocytes and natural killer cells (NK cells), CD3/19 and CD3/16/56 monoclonal antibodies were used. Active cells were detected by applying HLA-DR monoclonal antibodies. Antigen density within the cell was then estimated via mean fluorescence intensity. In order to quantitate the levels of fluorescence, the mean fluorescence intensity (MFI) of antigen-positive cells was calculated. To determine the fluorescence intensity of the stained cells, the logarithmic fluorescence channel intensity was converted to arbitrary units based on the Immuno Count 2.0 software. The MFI of the CD-positive histogram was measured from the upper limit of the negative control. Antigen-positive cells were compared to the appropriate FITC or PE-conjugated mouse IgG1/IgG2 control cells.

*2.8. Concentration of Soluble Fas in PF.* PF supernatants were collected immediately after centrifugation and stored at −700°C until analysis, not longer than 8 months. In the analysis, sFas concentration in the stored samples was measured with a sandwich enzyme-linked immunoassay (ELISA) kit (Chemicon, USA), according to the manufacturer's instruction. The data are shown as the mean values of duplicate samples. The sensitivity of the test was 0.1 U/l, and the range of standards was 0–15 U/l.

*2.9. Statistical Analysis.* The Statistica 5 test program was used to compare differences between the study and the reference groups. Data are expressed as means ± SD, and $p < 0.05$ was considered as significant.

## 3. Results

*3.1. Concentration of Immune Cells in PF and Percentage of Peritoneal Fluid Macrophages (CD45$^+$/CD14$^+$)*

*3.1.1. Immune Cell Concentration.* In women with endometriosis, significantly higher concentrations of PF leukocytes ($p < 0.001$) were found, in comparison to the reference group (Table 1). Moreover, the concentration of leukocytes in the PF of patients with stage I (2490 cells/mm$^3$ ± 1116) and stage II (2210 cells/mm$^3$ ± 1099) of endometriosis was significantly higher ($p < 0.001$ and $p = 0.003$, resp.) when compared to the reference group (1231 cells/mm$^3$ ± 420). No statistical difference ($p > 0.05$) was found in patients with endometriosis stage III/IV (1620 cells/mm$^3$ ± 377), in comparison to the reference group, but it is hard to draw conclusions from this result due to small sample of cases with advanced stages of endometriosis recruited to the study.

*3.1.2. Macrophages.* In patients with endometriosis, a significantly higher ($p < 0.0001$) percentage of PF cells expressing CD45$^+$/CD14$^+$ was found, as compared to the reference group. In addition, a significantly higher percentage of PF macrophages was detected in women with stages I and II ($p < 0.001$ and $p < 0.001$), but not in patients with stage III/IV ($p > 0.05$) endometriosis, when compared to the reference group (Table 1). However, the mean fluorescence intensity of CD14$^+$ antigen on PF macrophages did not differ in patients with endometriosis, when compared to the reference group (Table 1) and between patients with different stages of the disease.

Beyond the aforementioned, T-lymphocytes, CD3$^+$/CD19, and NK cells, CD3$^-$/CD16/56$^+$, in the PF of patients with endometriosis were found to be as much as 16.3% of all examined immune cells. This is in contrast to the same within 41.8% of all examined cells in the reference group. Moreover, B lymphocytes, CD3$^+$/CD19$^+$, were found to amount to less than 0.5% of the entire examined PF leukocyte population in both the study and the reference groups.

*3.2. Expression of HLA-DR (a Marker of Activation) on PF Mononuclear Cells.* In the endometriotic patients, a significantly higher ($p < 0.01$) percentage of PF macrophages expressing the HLA-DR antigen (HLA-DR$^+$ macrophages) was observed, as compared to the reference group: 85.4% ± 7.3 versus 62.2% ± 31.1, respectively. However, the expression of HLA-DR was significantly higher only in patients with stages I ($p = 0.02$) and II ($p = 0.04$) of endometriosis and did not differ in patients with stage III/IV of endometriosis ($p = 0.07$), when compared to the reference group (Table 2). Furthermore, the MFI of the HLA-DR antigen in macrophages obtained from endometriotic women was statistically higher ($p = 0.01$), as compared to the reference group: 148.0 ± 20.4 versus 129.3 ± 28.0, respectively. No significant differences were found when patients with different stages of the disease (I, II, and III/IV) were compared to the reference group (Table 2).

*3.3. Expression of CD95 (Fas) Antigen on PF Macrophages.* Fas (CD95$^+$) was expressed on 20.1% ± 15.7 of all observed macrophages, in patients with endometriosis, whereas only 6.6% ± 10.1 of all macrophages from the reference group expressed CD95 superficially (Figure 1). The observed differences in Fas expression were statistically confirmed in all stages of endometriosis. With regard to MFI, no statistical differences between study groups regardless of the stage of

Table 2: Soluble form of Fas (sFas) antigen concentration in peritoneal fluid and percentage of macrophages expressing CD95 ($CD95^+$) and HLA-DR ($HLA\text{-}DR^+$) antigens.

| | Endometriotic patients ($n = 26$) | Stage I ($n = 11$) | Stage II ($n = 10$) | Stage III/IV ($n = 5$) | Reference group ($n = 16$) |
|---|---|---|---|---|---|
| $CD95^+$ macrophages (%) | $20.1 \pm 15.7^*$ | $17.6 \pm 15.2^*$ | $16.6 \pm 7.8^*$ | $37.4 \pm 19.8^*$ | $6.6 \pm 10.1$ |
| MFI of CD95 antigen on macrophages | $101 \pm 17.5$ NS | $101.5 \pm 13.5$ NS | $108.9 \pm 17.8$ NS | $90.7 \pm 25.6$ NS | $98.9 \pm 24.2$ |
| $HLA\text{-}DR^+$ macrophages (%) | $85.4 \pm 7.3^*$ | $85.6 \pm 7.1^*$ | $84.0 \pm 7.7^*$ | $89.0 \pm 7.1$ NS | $62.2 \pm 31.1$ |
| MFI of HLA-DR antigen on macrophages | $148.0 \pm 20.4^*$ | $146.9 \pm 14.1$ NS | $145.8 \pm 14.6$ NS | $156.6 \pm 39.3$ NS | $129.3 \pm 28.0$ |
| sFas concentration in PF (U/l) | $9.0 \pm 7.4$ NS | $4.7 \pm 3.1$ NS | $9.9 \pm 7.4^*$ | $16.2 \pm 13.1^*$ | $5.5 \pm 4.4$ |

*Statistically significant in relation to the reference group. NS: statistically nonsignificant.

the disease (I, II, and III/IV) and the reference group were observed (Table 2).

*3.4. Soluble Fas Concentration in PF.* The concentration of the soluble form of Fas (sFas) in the PF of patients with II and III/IV stage endometrioses was $9.9 \pm 7.4$ and $16.2 \pm 13.1$, respectively, which was significantly higher than that found in the reference group: $5.5\ \text{U/l} \pm 4.4$ (Table 2).

## 4. Discussion

Numerous studies showed systemic and/or local (qualitative and quantitative) changes in patients with endometriosis. This is the first study concurrently evaluating the concentration/expression of opposite markers related to activation and apoptosis on PF macrophages (proliferative and proapoptotic) in patients with endometriosis and healthy controls. The immune background of endometriosis is well established, and much data regarding PF leukocyte components are available, yet some are contradictory. In the presented study, for example, significantly higher concentrations of macrophages in the PF of patients with endometriosis were found. This comprised more than 80% of all PF leukocytes. This observation is in agreement with data published by Weinberg et al., Haney et al., Halme et al., and Hill [21–24]. Contrary to our data, Awadalla et al. and Zeller et al. observed lower percentages of PF macrophages in women with endometriosis [25–27].

Interestingly, in several studies, in women with endometriosis, significantly higher concentrations of PF macrophages were found, even when compared to cases with abdominal infections. In the latter condition, a predominance of neutrophils has been observed (85% of PF leukocytes). The mechanism responsible for such cell distribution is still unknown.

Akoum et al. demonstrated that in women with endometriosis, peritoneal macrophages had an increased capacity to secrete MCP-1 (monocyte chemotactic protein-1) [27]. This mechanism might exacerbate peritoneal inflammation and promote the growth of endometrial implants. Tao et al. found that peritoneal MCP-1 plays an important role in the pathogenesis of infertility in the early stage of endometriosis [28]. In our study, the highest percentage of macrophages, as well as the highest level of leukocytes, was in the group of women with I and II stages of the disease. Such a situation could be the result of the mobilization of the local immune response in minimally advanced endometriosis. The question is still unanswered why the specific immunological response is developed only in the early stage of endometriosis and is weakened in more advanced stages. We put forward that there is a critical point in the natural history of endometriosis when self-defense mechanisms are switched on to avoid the autodestruction of the body.

Worth mentioning is the observation that in the group of women with endometriosis, macrophages did not only prevail amongst the immune cells in the peritoneal fluid but that more than 84% of these also activated and presented the expression of HLA-DR—a recognized activation marker of macrophages. Our data show that the expression of HLA-DR on PF mononuclear cells in women with endometriosis is significantly higher in stages I and II of the disease. We thus surmise that cell-mediated immunity is the first line of immune response in endometriosis, but still it is not obvious if this reaction is sufficient to obtain peritoneal homeostasis. It, hence, may only indirectly take part in the antigen presentation to T cells.

Activated macrophages are capable of secreting several biological substances. This process could be responsible for inducing the growth of endometriotic lesions in women with endometriosis. This theory tends to support the works of Halme et al., Koutsilieris et al., Olive et al., and Hammond et al. [29–32]. They held that PF macrophages secrete biological substances which promote the proliferation of endometrial cells. Moreover, concentrations of this substances increase with the advance of the disease [33, 34]. The expression of HLA-DR on macrophages is crucial for the induction of a humoral immune response. In accordance with our observation that in the group of women with endometriosis, the percentage of activated macrophages was twofold higher than that in the control group, we opine that the function of macrophages is also increased. Still, many authors have found a dysfunction of PF macrophages in endometriosis, but the etiology is of yet unknown [9, 35, 36]. Dmowski et al. suggest that this results from a

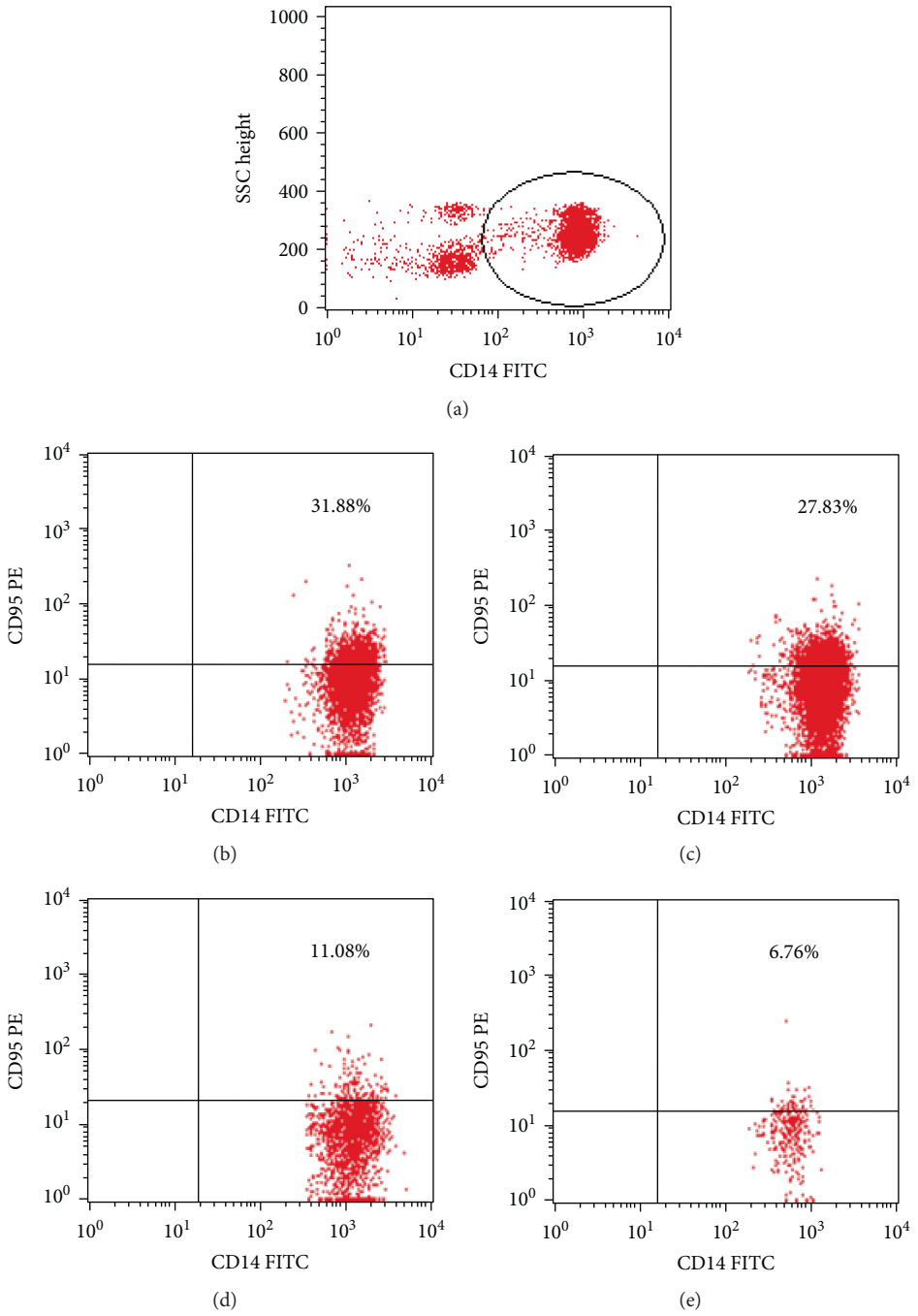


Figure 1: Representative dot plots, illustrating our analysis method for the identification of $CD14^+$ cells with CD95 (Fas) expression. (a) An acquisition gate was drawn around the $CD14^+$ cells. (b–e) Next, the gated events were analyzed for CD95 PE. The dot plots (b–d) show representative types of CD95 expression in $CD14^+$ cells from patients with endometriosis. The dot plot (d) shows the representative type of $CD14^+CD95^+$ cells from the reference group.

congenital or acquired defect of the immune system. Of note, in recent years, many studies have seen the inhibiting influence of environmental or immunosuppressant substances in the PF of women with endometriosis [8].

Many authors have also emphasized the dual role of macrophages in the pathogenesis of the disease. Firstly, they intensify the immune response leading to the elimination of pathologic lesions. Secondly, and in contrast, they promote the growth of endometriotic implants. These two processes form a vicious circle and could be responsible for the developing of endometriosis. Further studies of this issue, however, are still required.

Many studies have also evaluated the role of immune activation in women with endometriosis (i.e., bcl- and bax-gene activation in PF macrophages); however, only a few studies have dealt with the issue of macrophage apoptosis

in endometriosis and have assessed Fas antigen expression on PF macrophages in the disease [37, 38]. Our study generated an estimate of the presence of the Fas antigen on the macrophage surface.

Some authors have suggested that proapoptotic receptors and their ligands on the surface of immune and endometrial cells play crucial roles in the pathogenesis of endometriosis [39]. In the presented study, in the group of women with endometriosis, the level of the Fas receptor was statistically higher than that in the control group. Moreover, the expression of the Fas receptor increased with the stage of the disease. Indeed, the percentage of PF macrophages with the Fas receptor in patients at stage III/IV of endometriosis was more than twofold higher than that in stage I. This situation could be the result of a higher concentration of TNF$\alpha$ in the peritoneal fluid of women with endometriosis [40–44].

The presence of TNF$\alpha$ upregulates the Fas receptor in the cells, and it brings about Fas-induced apoptosis. We suspect that activated macrophages are the source of TNF$\alpha$ which begins a cascade of events finally leading to the increased synthesis of IFN-$\gamma$. This, on the one hand, enhances the cytotoxicity of NK cells and macrophages and, on the other hand, promotes cell self-destruction.

Garcia-Velasco et al. found a stage-dependent decreased expression of the Fas ligand (FasL) on the ectopic endometrium in women with endometriosis [7]. The expression, they put forward, was induced by dose-dependent macrophage-derived factors such as TGF-$\beta$ and PDGF. Furthermore, they held that the interaction between endometrial cells and the extracellular matrix (which contains laminin, fibronectin, and collagen IV) upregulates FasL expression. This may protect the endometriotic cells from attack by activated T lymphocytes, and the Fas-FasL mechanism may also allow eutopic endometrial cells to escape from immune surveillance. The lower activity and concentration of the immune cells in the peritoneal fluid of women with endometriosis appear to confirm this scenario [25, 26, 45].

It is worth mentioning about the effect of Danazol and the aromatase inhibitors on the immune system in endometriosis. Such drugs normalize the peritoneal concentration of TNF$\alpha$ and increase the number and cytotoxicity of immune cells acting against the ectopic endometrium [1]. In our work, at the advanced stages of endometriosis, a significantly higher percentage of PF macrophages with Fas expression was observed (accompanied by the lowered concentration of soluble forms of the receptor), but the reliability of this finding is limited by the small number of analyzed cases with advanced endometriosis.

Our data show that the level of soluble forms of Fas in the group of women with stages II and III/IV of endometriosis is statistically higher than that in the reference group. The same observation was made by Linghu et al. [46]. In a related work, the concentration of sFasL in the serum and peritoneal fluid of women with moderate to severe endometriosis was elevated when compared to that of women with minimal and mild endometriosis [47]. In this study, the sources of soluble FasL in the peritoneal fluid were thought to be the ectopic endometrial cells and the peritoneal leukocytes. Still, other studies report that matrilysin (uterine metalloproteinase) generates the active soluble form of FasL [48, 49].

The presence of both forms of the Fas ligand (soluble and membrane-bound) in the peritoneal fluid of women with endometriosis seen in cited works, in comparison with our results, may suggest increased immune cell apoptosis in the peritoneal cavity. The deficiency of immune cells, therefore, must lead to local immune dysfunction, to incomplete immune response, and, consequently, to the development of the disease. The significantly higher percentage of macrophages expressing the Fas receptor supports the hypothesis that the PF components of women in endometriosis may promote the development of the disease.

## 5. Conclusion

An imbalance between apoptosis and activation/surveillance/proliferation of immune and endometrial cells may play an important role in the pathogenesis of endometriosis.

The high number of immune cells in the peritoneal fluid and their increased susceptibility to apoptosis in early-stage endometriosis emphasize the role played by both an impaired peritoneal environment and immune defects in the development of the disease.

## Conflicts of Interest

None of the authors have a conflict of interest.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 4809294, 4 pages
https://doi.org/10.1155/2017/4809294

*Research Article*

# An Exploration of the Impact of Anticentromere Antibody on Early-Stage Embryo

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Received 11 June 2017; Accepted 16 July 2017; Published 4 October 2017

Academic Editor: Jacek Tabarkiewicz



*Background.* Previously, we found women with positive anticentromere antibody showed impaired potential of oocyte maturation and embryo cleavage; the possible mechanism behind this phenomenon was still unknown. *Objective.* Thus, the present study aimed to preliminarily explore whether ACA could penetrate into the living embryos and impair their developmental potential via *in vitro* coculture with mouse embryos. *Methods.* Mouse embryos were collected and used for *in vitro* culture with polyclonal anticentromere protein A (CENP-A) antibody; then, immunofluorescence assay was performed to determine the penetration of antibody into embryos, and embryo development potential was observed. *Results.* All embryos cultured with anti-CENP-A antibody exhibited immunofluorescence on the nucleus, while none of the embryos from the control groups showed immunofluorescence. Additionally, embryos cultured with anti-CENP-A antibody experienced significant growth impairment compared with controls. *Conclusion.* Mouse embryos may be a direct target for ACA *in vitro* prior to implantation. However, the precise mechanism needs further clarification.

## 1. Introduction

A recent study revealed disorders of oocyte maturation and early embryonic development in women with positive anticentromere antibody (ACA) in their peripheral blood [1]. More recently, we found that women positive for ACA had a significantly lower percentage of mature oocytes and embryo cleavage rate compared with women negative for ACA [2], further revealing the potential impact of ACA on female fertility. ACA is known to be one of the members of ANAs. It was first discovered in 1980 as a specific antibody against centromere in serum of patients with calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia (CREST) syndrome [3, 4]. Now, ACA has been recognized as an effective auxiliary diagnostic marker for systemic sclerosis (SSc). As reported, female patients with SSc are susceptible to have several different adverse pregnancy outcomes, including increased spontaneous abortion rate, premature birth, small babies, and infertility [5, 6]. Additionally, the infertility prevalence in patients with SSc is high, and the success rate for infertility treatment is relatively low, which needs further investigation [7].

As early as the 1990s, researchers attempted to microinject ACA into mouse eggs, which led to disorders of chromosomal movement and segregation [8]. It is known that kinetochore is the attachment site of spindle microtubules in the centromeric region of a chromosome [9, 10]. Also, it is the dynamic structure for mitosis, meiosis, and other important activities of cells [11–15]. Therefore, it would be reasonable to infer that ACA might interfere with meiosis or mitosis in living cells.

Centromere is a DNA-protein complex, and its assembly is coregulated by centromeric chromatins and their associated protein complex [16, 17]. Centromere protein A (CENP-A) is one of the constitutive centromere proteins with relatively clear biological functions that has been mostly studied; its important role in assembly and functional implementation of centromere has been repeatedly verified [18, 19]. Furthermore, similar to CENP-B, CENP-A is considered to be a major target antigen of ACA [20–23].

It was speculated that ACA might be one of the ANAs most closely associated with abnormal oocyte maturation and embryo cleavage. Therefore, the aim of the present study was to explore the potential impact of ACA on early-stage embryos via *in vitro* coculture with mouse embryos.

## 2. Materials and Methods

*2.1. Mouse Embryos.* Superovulation was induced in outbred ICR mice by stimulating with pregnant mare's serum gonadotrophin (10 IU intraperitoneally (i.p.)) and human chorionic gonadotrophin (10 IU i.p. after 48 h) and mated with ICR males. The female mice were killed 24 h after mating. Early-stage embryos were collected by sharp dissection of the fallopian tubes and used in the experiments. The Ethics Committee of the First Affiliated Hospital of Sun Yat-Sen University approved this study.

*2.2. In Vitro Embryo Culture.* The embryos were cultured in the Quinn's serial medium (Sage, USA). For the antibody group, rabbit polyclonal antibody to mouse CENP-A (bovine serum albumin and azide free, customized products from Abcam, United Kingdom) was added to the medium. The antibody concentration in the medium was 35 $\mu$g/mL (modified based on the literature [24]). For the phosphate-buffered saline (PBS) group (served as controls), the PBS solution (PBS tablet, Millipore, Merck, Germany) with the same volume as the antibody solution was added to the medium. The blank control group comprised the medium without any additives.

*2.3. Immunofluorescence Assay.* On the second and third days of culture, three to five embryos were picked from each dish of the three groups for the immunofluorescence assay, to detect whether the signals of anti-CENP-A antibody were present in the embryos after coculture. The procedures for the immunofluorescence assay were as follows: The embryos were fixed in 4% polyoxymethylene and then permeated with 0.5% Triton X-100 (Sigma, USA), followed by sealing in 5% normal donkey serum (Jackson Immunoresearch, USA). After that, the embryos were incubated for 1 h with 488-labeled donkey antirabbit IgG (Invitrogen, UK, 1 : 1000 dilution), rinsed, incubated with 1 $\mu$g /mL DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride, Cell Signaling Technology, USA) for 15 min, again rinsed, and fixed in a dish for subsequent microscopic observation. In order to rule out false positive of the experimental group, embryos from antibody group were incubated with PBS instead of 488-labeled donkey antirabbit IgG (antibody group for control).

*2.4. Development of Cocultured Embryos (Embryotoxicity Assay).* The collection and culturing of the embryos for this embryotoxicity assay were the same as described earlier except that the embryos remained in the dishes for the entire 3-day period. The embryos in each group were examined to determine their stage of development on the third and fifth days (the first day referred to the day of oocyte collection). The following developmental stages were recorded: 6- to 8-cell stages on the third day, and blastocyst, morula, 2- to 8-cell, and atretic stages on the fifth day.

*2.5. Statistical Analysis.* Statistical analysis was performed using SPSS 13 (SPSS, IL, USA). A chi-square test and partition of chi-square tests were used to compare qualitative data. A $P$ value less than 0.05 was considered statistically significant by chi-square test among the three groups, and a $P$ value less than 0.0167 was used to indicate statistical significance in the partition of chi-square tests between groups.

## 3. Results

*3.1. Immunofluorescence.* All embryos cultured with anti-CENP-A antibody exhibited strong immunofluorescence in their nuclei, while none of the embryos from the PBS and blank control groups, as well as the antibody group for control, showed immunofluorescence (Figure 1).

*3.2. Embryotoxicity Assay.* Compared with the PBS and blank control groups, the percentages of 6- to 8-cell stage embryos on the third day, as well as blastula and morula stage embryos on the fifth day, were significantly lower in the antibody group. The developmental potentials of embryos were comparable between the PBS and blank control groups (Table 1).

## 4. Discussion

In 1999, researchers found that all mouse embryos cultured with purified antinuclear IgG exhibited strong immunofluorescence on the embryonic cells and experienced significant growth impairment or death compared with those cultured with control immunoglobulins [25]. This indicated that ANAs could bind directly to embryos *in vitro*. However, the precise epitopes were not known because no nuclear antigens or phospholipids were found in the zona. In mouse 2-cell stage embryos, a set of nucleoproteins is transiently synthesized and changes in embryonic chromatin composition, suggesting that early embryos may possess epitopes for ANA [26]. Furthermore, the binding is relatively specific, as antithyroid antibody and antibodies from healthy individuals show no evidence of binding to embryos. Microinjection of serum containing ACA into mouse oocytes could hinder chromosome congression and cause meiotic arrest in interphase or mitotic arrest in prometaphase [8].

In the present study, all embryos cultured with polyclonal anti-CENP-A antibody showed strong immunofluorescence of antibody against nuclear components (which were speculated to be anti-CENP-A antibody) and experienced apparent embryonic growth impairment, indicating that mouse embryos may be a direct target for some ACAs *in vitro* prior to implantation. In addition, for the majority of cocultured embryos, always only one or some of the blastomeres showed fluorescence. Perhaps, the density of structures in and around the centromere prevents anti-CENP-A antibody accessibility, or the blastomere with detectable fluorescence was inclined to apoptosis and displayed relatively loose structures that enabled anti-CENP-A antibody accessibility. However, the precise mechanism needs further clarification.

Although no definite concept exists for antibody entering the living cells, and the mechanism involved is unknown yet, these studies provided evidence for antibodies entering the

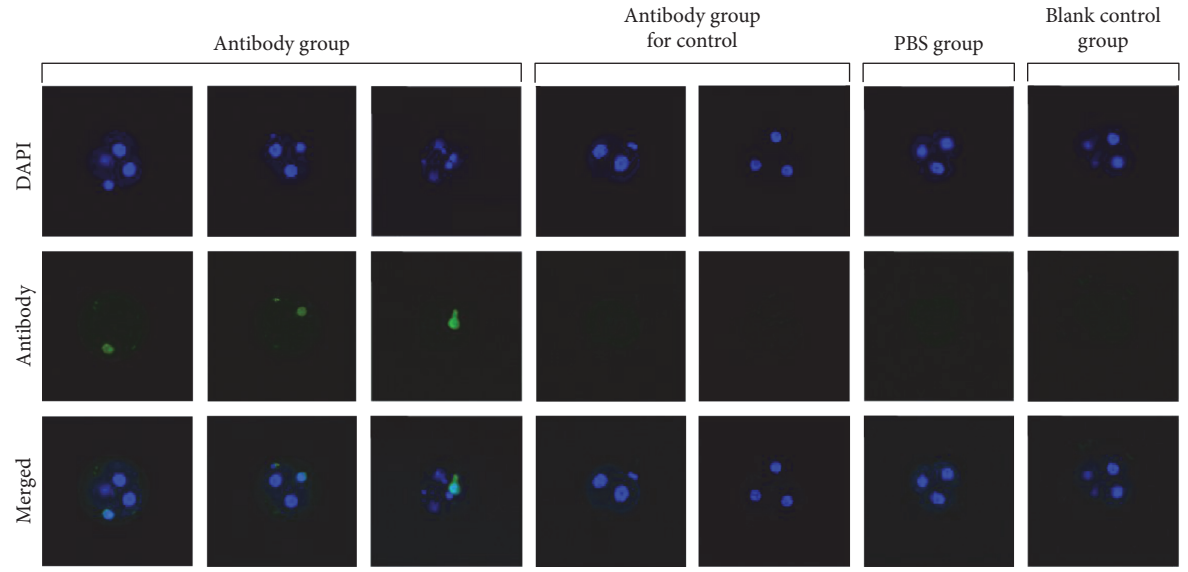


Figure 1: Immunofluorescence views of embryos cultured with anti-CENP-A antibody (original magnification ×400). Antibody fluorescence was present on the nuclei of blastomeres in embryos from the antibody group, while no significant antibody fluorescence was observed in the embryos from groups of PBS, blank control, and antibody group for control.

Table 1: Embryotoxicity assay.

| Parameters (%) | Antibody group | PBS group | Blank control group | *P* value |
|---|---|---|---|---|
| 6- to 8-cell stages on the third day | 24.8% (114/459) | 48.4% (183/378) | 51.0% (192/376) | <0.01[a,b] |
| Blastula stage on the fifth day | 26.8% (123/459) | 57.1% (216/378) | 64.9% (244/376) | <0.01[a,b] |
| Morula stage on the fifth day | 12.4% (57/459) | 24.6% (93/378) | 20.2% (76/376) | <0.01[a,b] |

The first day referred to the day of embryo collection. $P < 0.05$ was considered statistically significant among the three groups. [a]$P < 0.0167$ versus antibody and PBS groups. [b]$P < 0.0167$ versus antibody and blank control groups.

living cells. For example, the anti-ribonucleoprotein-IgG could selectively enter the T-lymphocytes, while relatively less Fc receptors were present on the surface of T-lymphocytes. It suggested that some other mechanisms relevant to non-Fc regulation might be involved, which might be associated with antigen-like structures on the lymphocyte surface, implying that the ribonucleoprotein antibodies interacted with the ribonucleoprotein antigens on the cell surface to regulate their access into the living cells [27].

This study also found that the embryonic developmental potential was significantly impaired after culture with anti-CENP-A antibody, exhibiting significantly lower percentages of 6- to 8-cell stage embryos on the third day and blastula stage embryos on the fifth day. In fact, a previous study found that mouse embryos cultured with purified IgG from ANA-positive serum showed significantly impaired embryonic development potential [25]. ANA could penetrate into subcellular structures containing corresponding antigens, where it could identify and bind to epitopes in the important functional regions. These autoantibodies could inhibit significantly the function of antigens both in vivo and *in vitro* [28].

In conclusion, embryos cultured with anti-CENP-A antibody experienced significant growth impairment or death. Thus, embryos could be a direct target for anti-CENP-A antibody.

## Ethical Approval

Medical Ethics Committee of The First Affiliated Hospital of Sun Yat-sen University approved the study (no. [2014]75).

## Conflicts of Interest

Ying Ying, Xi Guo, Yiping Zhong, and Canquan Zhou declare that they have no conflict of interest.

## Acknowledgments

This study was supported by the National Natural Science Foundation of China (no. 81701518), Guangzhou Science and Technology and Information Bureau (no. 2014J4100161), Science & Technology Project of Guangdong Province (no. 2013B021800271), and Population and Family Planning Commission of Guangdong Province (no. 20132048). This study was supported by the Key Laboratory for Reproductive Medicine of Guangdong Province from The First Affiliated Hospital of Sun Yat-sen University. The study

was also supported by two key laboratories from The Third Affiliated Hospital of Guangzhou Medical University; they were the Key Laboratory for Major Obstetric Diseases of Guangdong Province and Key Laboratory of Reproduction and Genetics of Guangdong Higher Education Institutes.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 8245879, 9 pages
https://doi.org/10.1155/2017/8245879

*Research Article*

# Maternal and Perinatal Outcome in Women with Systemic Lupus Erythematosus: A Retrospective Bicenter Cohort Study

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Received 26 May 2017; Revised 28 July 2017; Accepted 8 August 2017; Published 28 September 2017

Academic Editor: Jacek Tabarkiewicz



*Objective*. To investigate disease activity around and during pregnancy and pregnancy outcome in women with systemic lupus erythematosus (SLE) considering antiphospholipid antibody status. Moreover, differences between first and consecutive pregnancies were examined. *Methods*. Pregnancies > 16 weeks gestation of SLE patients receiving joint care from rheumatologists and gynecologists in two tertiary centers in the Netherlands between 2000 and 2015 were included. Disease activity, flare rate, and pregnancy outcomes and complications were assessed. *Results*. Ninety-six women (84% Caucasian) with 144 pregnancies were included. The median SLE(P)DAI score was 2 before, during, and after pregnancy. Flare rates were 6.3%, 20.1%, and 15.3%, respectively. Severe hypertensive disorder of pregnancy, intrauterine fetal death, preterm birth, and small-for-gestational age infants occurred in 18.1%, 4.1%, 32.7%, and 14.8%, respectively. Complication rates were similar in the first and consecutive pregnancies. Half of the women did not experience any pregnancy complication whereas 42.7% developed a complication during all pregnancies. Mean number of pregnancies was 2.4 and live births 1.7. *Conclusion*. In this SLE population with low disease activity, pregnancy complications were present irrespective of antiphospholipid antibody status. Furthermore, there were no differences in complication rates between the first and consecutive pregnancies as seen in healthy mothers. This information is useful for patient counseling.

## 1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that often affects women during their childbearing age [1]. It is well known that women with SLE may experience an increase in disease activity during pregnancy [2–4]. Moreover, women with SLE have a higher risk of experiencing pregnancy complications like hypertensive disorders (HD) of pregnancy (pregnancy-induced hypertension (PIH); preeclampsia; eclampsia; and hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome), preterm birth, intrauterine fetal death (IUFD), and small-for-gestational age (SGA) infants compared to the general population [4–7].

Several risk factors for pregnancy complications in women with SLE have been reported, amongst them are the

presence of antiphospholipid antibodies (aPL) or antiphospholipid syndrome (APS), (prior) lupus nephritis, and active disease at conception [8–12]. Therefore, low disease activity for at least six months is recommended to lower the risk for SLE flares and maternal and perinatal complications [13–16]. In order to achieve this, preconceptional counseling and close collaboration between gynecologist and rheumatologist are recommended [10, 13, 17]. Evaluation of risk factors (e.g., smoking, hypertension, overweight, and family history) and optimization of timing of pregnancy are goals of preconceptional counseling. Moreover, the use of pregnancy compatible medication, amongst others azathioprine and hydroxychloroquine (HCQ), is evaluated in order to prevent flares and maternal and perinatal complications [18].

Over the last decades, an improvement in pregnancy outcomes in SLE patients has been reported [19]. A recent large North-American multicenter study investigated one pregnancy per woman with SLE ($n = 385$), excluding patients with comorbidity such as diabetes or impaired renal function and patients using medium or high-dose glucocorticosteroids. The results of this study demonstrated that 80% of the neonates was born alive after a gestation period >36 weeks, not including miscarriages [20].

In the present study, pregnancies of women with SLE over a 16-year period, irrespective of comorbidity and medication use, are described.

In the general population, HD and PIH occur most commonly in the first pregnancies [21]. This has not been examined yet in a population with SLE, where several factors (e.g., underlying immune activation, impaired renal function, or APS) might be associated with a higher incidence of HD and other pregnancy complications also in consecutive pregnancies.

The aim of the present study is to examine three topics, taking the antiphospholipid antibody status into account:

(1) SLE disease activity before, during, and after pregnancy per pregnancy

(2) Maternal and perinatal complications occurring in the first and consecutive pregnancies and during the reproductive period

(3) Total number of live births per patient

The results of this study will provide relevant information for health care professionals who are involved in the treatment and preconceptional counseling of these patients and their partners.

## 2. Patients and Methods

This cohort study involved two tertiary centers in the Netherlands: the University Medical Center Utrecht and the VU University Medical Center in Amsterdam. To identify pregnancies in women with SLE, a search was performed in both obstetric and rheumatology databases. Data were retrieved from medical files and collected in both centers using the same case report form. The Institutional Review Boards of both university hospitals concluded that official approval from a medical ethical committee was not needed due to the observational character of this study.

*2.1. Participants.* Inclusion criterion was diagnosis of SLE according to the American College of Rheumatology (ACR) revised criteria [22], diagnosed before the start of the first recorded study pregnancy. To contain uniformity in the classification of SLE, only the ACR revised criteria were used for all pregnancies, even though in 2012 new SLE classification criteria were published [23]. Moreover, only patients with both obstetric and rheumatology check-ups during pregnancy in one of the two participating centers were included. All ongoing pregnancies (>16 weeks of gestation) between the years 2000 and 2015 were included. No exclusion criteria were formulated.

Antiphospholipid antibody status was recorded in all patients. Patients were divided into SLE without aPL, SLE with aPL, or SLE with APS. Presence of aPL was defined as two positive measurements of either IgG or IgM anticardiolipin antibodies or lupus anticoagulant, measured at least six weeks (before 2006) or 12 weeks (after 2006) apart and, when applicable, not during pregnancy or within ten weeks thereafter [24, 25]. In 29.9% of the pregnancies, presence of beta-2-glycoprotein antibodies was measured as well. Samples were considered positive for anticardiolipin antibodies or beta-2-glycoprotein antibodies when either above 40 GPL, 40 MPL, or above the 99th percentile. APS was diagnosed according to the Sapporo criteria [24].

*2.2. Outcomes.* Baseline characteristics included information about aPL status, demographic background, age, body mass index (BMI), and general and obstetric history. The obstetric history included miscarriages (<16 weeks gestation), severe HD (preeclampsia, eclampsia, and HELLP syndrome), IUFD, preterm birth (<37 weeks), and SGA infants (birth weight < p10).

Disease activity was assessed in retrospect using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) within 6 months before pregnancy and within 6 months postpartum [26]. In each trimester of pregnancy, disease activity was assessed using SLEPDAI (SLEDAI adjusted for pregnancy) [27]. Flares were defined according to the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) SLEDAI definitions [28]. Clinical manifestations of SLE (according to the ACR revised criteria [22]) and (changes in) dosages of medication were registered. Anticoagulation therapy during pregnancy of this population has been published [29].

The following maternal and perinatal complications were scored: mild HD (PIH), severe HD, IUFD, preterm birth (both <36 and <37 weeks gestation), SGA infants, and occurrence of neonatal lupus: either cutaneous lupus or congenital heart block. Occurrence of HD (and thereby distinction with nephritis) was scored by one of the gynecologists (ATL and JIPdV).

In the first analysis, maternal and perinatal complications were described for all pregnancies meeting the inclusion criteria; secondly, a comparison between the first and consecutive pregnancies meeting the inclusion criteria was

undertaken. In the third analysis, all complications per included patient during the studied reproductive period were examined. The latter was defined as the study pregnancies during the 16-year period and prior obstetric history.

Data are presented per total SLE population and per any of the three subdivisions: aPL absent, aPL present but not fulfilling APS criteria, and aPL present plus fulfillment of APS criteria.

Total number of pregnancies, total number of live births, and miscarriage rate per patient were examined during the studied reproductive period.

*2.3. Statistics.* Baseline characteristics were examined per antiphospholipid group using Fisher's exact test or chi-square test for dichotomous variables and independent samples median test or one-way ANOVA for continuous variables.

Differences in disease activity and start or increase of prednisone, azathioprine, and HCQ dose between the three antiphospholipid groups were tested using independent samples median test for continuous variables or Fisher's exact test for dichotomous variables. Total number of flares during pregnancy compared with the total number of flares postpartum was examined using a Fisher's exact test.

Differences in incidence rates of maternal and perinatal complications between the three aPL subdivisions were investigated using generalized estimating equations, for which an exchangeable correlation structure was chosen. This analysis corrects for patient dependency since some women in our cohort were included with multiple pregnancies. All outcomes were corrected for smoking, body mass index (BMI) >25 $kg/m^2$, and prednisone use.

Differences in maternal and perinatal complication rates between the first and consecutive pregnancies were examined using a chi-square test or Fisher's exact test.

Maternal and perinatal complication rates and numbers of live births in the studied reproductive period were studied using descriptive statistics.

Statistical analysis was performed using SPSS for Windows (version 22, SPSS Inc., Chicago, IL, USA). A two-sided $p$ value inferior to 0.05 was considered to be statistically significant.

## 3. Results

*3.1. Baseline Characteristics.* In total, 96 patients with 144 pregnancies met the inclusion criteria. Distribution of the parity was 70 nulliparous women and 18 primiparous women, 7 women were para 2, and 1 woman was para 4 at the first included study pregnancy. Baseline characteristics are presented in Table 1. In the group of 10 patients with SLE and APS, nine had a history of thrombotic APS and four had a history of obstetric APS. LAC status was positive in 28.6% of the patients with SLE and aPL and positive in 84.6% of the patient with SLE and APS. Of the non-Caucasian patients, eight were black and seven women were Asian. Thirty-three percent of all patients had a BMI above 25 ($kg/m^2$). None of the patients had a platelet count below $100 \times 10^9$/L at the start of the pregnancy. The only significant difference in baseline characteristics between groups was the inherent significantly increased history of thrombosis in APS-diagnosed women.

*3.2. SLE Manifestations and Disease Activity before, during, and after Pregnancy*

*3.2.1. ACR Criteria.* Most frequently found ACR criteria contributing to the diagnosis of SLE before pregnancy were arthritis, renal involvement, positive ANA, and hematological (leuco-, lymphocyto-, or thrombocytopenia, hemolytic anemia) and/or immunological (anti-dsDNA, anti-Smith, or anticardiolipin antibodies or LAC) anomalies. There were no differences in prevalence of the ACR criteria between both centers (data not shown). Arthritis was significantly less often found in the SLE + APS group ($p = 0.01$); the other ACR criteria did not show statistically significant differences between the groups.

*3.2.2. Disease Activity and Flares.* Results of disease activity measurements according to the SLE(P)DAI criteria before and during pregnancy and postpartum are presented in Table 2. Only the SLEPDAI score during the 3rd trimester was significantly lower in the SLE-aPL group compared to the other groups ($p < 0.01$). In 30.6% of pregnancies, a flare occurred during the pregnancy period or within six months before or after the pregnancy. Flares were mostly characterized by decreased complement levels, hematuria, proteinuria, rash, or arthritis. Of nine pregnancies in which a mild flare occurred within six months before pregnancy, five experienced consecutive flares either during pregnancy ($n = 1$), postpartum ($n = 3$), or both ($n = 1$). Severe flares occurred three times during pregnancy and twice postpartum. These severe flares were characterized by (amongst others) nephritis, pleuritis, and rash. One patient had both mild flares (during pregnancy) as well as a severe flare (postpartum).

Flare rates postpartum were lower than during pregnancy (15.3 versus 20.1% resp., $p < 0.01$). Out of 22 pregnancies in which a flare occurred postpartum, 14 also had a flare before or during pregnancy (63.6%, $p < 0.01$). In total, 61 flares occurred in 44 pregnancies.

*3.2.3. Medication Use.* Treatment with prednisone, azathioprine, or HCQ was started or dosages were increased during pregnancy in 17%, 4%, and 3% of the pregnancies, respectively (Table 2). Frequencies of initiation or dose increase of prednisone or azathioprine during pregnancy did not differ per antiphospholipid group ($p = 0.77$ and $p = 0.72$, resp.). Initiation or dose increase of HCQ during pregnancy was more frequent in patients with SLE + APS compared to the other two groups ($p < 0.01$).

In 54 pregnancies, HCQ was used. Comparing the treatment before and after 2008, the use of HCQ during pregnancy increased: 16% received HCQ before 2008 and 58% after 2008 ($p < 0.01$). Flare rate during pregnancy ($p = 0.09$), occurrence of severe HD ($p = 0.31$), IUFD ($p = 0.20$), or preterm birth < 37 weeks ($p = 0.75$) did not differ before and after 2008.

Table 1: Baseline characteristics per study pregnancy.

| Pregnancies ($n$) | Total 144 | SLE-aPL 117 | SLE + aPL 14 | SLE + APS 13 | $p$ value |
|---|---|---|---|---|---|
| Number of women | 96 | 77 | 9 | 10 | NA |
| Study pregnancies per woman | 1 [1-2] | 1 [1-2] | 1 [1-2] | 1 [1–1.5] | 0.71 |
| Non-Caucasian[#] | 15/91 (16.5) | 13/74 (17.6) | 2/8 (25.0) | 0/9 (0) | 0.36 |
| Age (years) | 31.9 ± 4.4 | 32.1 ± 4.4 | 29.7 ± 4.0 | 32.5 ± 4.7 | 0.16 |
| BMI (kg/m$^2$) | 23.7 ± 4.4 | 23.2 ± 3.5 | 25.2 ± 3.6 | 25.9 ± 9.0 | 0.05 |
| Smoking | 12/139 (8.6) | 10/113 (8.8) | 0/14 (0) | 2/12 (16.7) | 0.27 |
| *General history* | | | | | |
| Chronic hypertension | 20/142 (14.1) | 16/117 (13.7) | 2/13 (15.4) | 2/12 (16.7) | 0.81 |
| Diabetes | 5/143 (3.5) | 4/117 (3.4) | 0/14 (0) | 1/12 (8.3) | 0.42 |
| History of thrombosis[*] | 23/144 (16.0) | 14/117 (12.0) | 0/0 (0) | 9/13 (69.2) | <0.01 |
| Serum creatinine level < 6 months before pregnancy ($\mu$mol/L) | 67.2 ± 11.4 | 67.6 ± 10.9 | 69.0 ± 13.4 | 62.6 ± 13.2 | 0.31 |
| *SLE-specific history* | | | | | |
| SLE duration before start pregnancy (years) | 7.8 ± 4.9 | 7.7 ± 5.0 | 8.7 ± 4.2 | 7.6 ± 4.6 | 0.76 |
| History of nephritis[^] | 57/144 (39.6) | 45/117 (38.5) | 7/14 (50.0) | 5/13 (38.5) | 0.70 |
| SS-A and/or SS-B positive | 70/138 (50.7) | 61/111 (55.0) | 6/14 (42.9) | 3/13 (23.1) | 0.08 |
| *Medication use at start pregnancy* | | | | | |
| Hydroxychloroquine | 69/135 (51.1) | 54/109 (49.5) | 6/13 (46.2) | 9/13 (69.2) | 0.38 |
| Azathioprine | 39/140 (27.6) | 35/114 (30.7) | 3/13 (23.1) | 1/13 (7.7) | 0.21 |
| Prednisone | 74/140 (52.9) | 63/114 (55.3) | 7/13 (53.8) | 4/13 (30.8) | 0.25 |
| *Obstetric history* | | | | | |
| Miscarriages[∞] | 32/94 (34.0) | 26/78 (33.3) | 2/8 (25.0) | 4/8 (50.0) | 0.68 |
| Severe HD | 19/63 (30.2) | 16/51 (31.4) | 2/7 (28.6) | 1/5 (20.0) | 1.00 |
| IUFD | 13/91 (14.3) | 13/76 (17.1) | 0/8 (0) | 0/7 (0) | 0.33 |
| Preterm birth (<37 weeks) | 24/76 (31.6) | 21/64 (32.8) | 2/7 (28.6) | 1/5 (20.0) | 1.00 |
| SGA infant | 17/69 (24.6) | 16/57 (28.1) | 0/14 (0) | 1/5 (20.0) | 0.34 |

Data depicted as numbers (%), mean ± standard deviation, or median [interquartile range]. [#]This item is depicted per woman, not per pregnancy, [*]either arterial or venous, [^]biopsy proven, [∞]<16 weeks gestation. SLE: systemic lupus erythematosus; aPL: antiphospholipid antibodies; APS: antiphospholipid syndrome; NA: not applicable; BMI: body-mass index; severe HD: hypertensive disorders of pregnancy including preeclampsia, eclampsia, and HELLP syndrome; IUFD: intrauterine fetal death; SGA: small-for-gestational age (birth weight < p10).

*3.3. Maternal and Perinatal Complications.* Maternal and perinatal complications of all study pregnancies are presented in Table 3. In total, there were three twin pregnancies.

*3.3.1. Maternal Complications.* HELLP syndrome was found statistically significantly more often in the SLE + APS group compared to the other groups ($p < 0.01$). A placental abruption occurred in one pregnancy in a patient with SLE without aPL. In 36.8% of all pregnancies, a caesarian section was conducted, which was not different between the groups ($p = 1.00$).

*3.3.2. Perinatal Complications.* Of all preterm births (<37 weeks), 44.2% occurred spontaneously, and in the others, labour was induced. Main indications for preterm induction of labour (<37 weeks) were HD (54.1%) and IUFD (12.5%). Of all pregnancies, 32.7% ended before 37 weeks and 24.3% before 36 weeks. Of all live-born infants, 55.3% were admitted to the medium care or neonatal intensive care unit. No neonatal deaths occurred. Of the two infants with neonatal lupus, one had a congenital heart block and the other cutaneous lupus. Congenital anomalies occurred in nine pregnancies of which the overwhelming majority [8] occurred within the SLE-aPL group, albeit not statistically significant ($p = 0.06$). Anomalies included trisomy 21 with Fallot's tetralogy, premature retinopathy, omphalocele, macrosomia, clubfoot, anus atresia, hip dysplasia, congenital (bilateral) glaucoma (2 children), and muscular ventricular septal defect. The congenital glaucoma occurred in siblings.

*3.3.3. Pregnancy Complications in the First and Consecutive Pregnancies.* The incidence of pregnancy complications did not differ between the first ($n = 70$) and consecutive ($n = 74$) pregnancies, with severe HD occurring in both first and consecutive pregnancies in 18.6% and 17.6%, respectively ($p = 0.88$), preterm birth < 37 weeks in 36.6% and 28.9%, respectively ($p = 0.32$), IUFD in 4.2% and 3.9%, respectively ($p = 1.00$) and SGA in 14.7% and 14.9%, respectively ($p = 0.98$). The rates of maternal and perinatal pregnancy complications for the studied reproductive period per patient are presented in Figure 1. Complications mostly

Table 2: Disease activity before, during, and after pregnancy and medication alterations.

| | Total ($n = 144$) | SLE-aPL ($n = 117$) | SLE + aPL ($n = 14$) | SLE + APS ($n = 13$) | $p$ value |
|---|---|---|---|---|---|
| SLEDAI < 6 months before pregnancy | 2 [0–4] | 2 [0–4] | 2 [0–4] | 3 [2–4] | 0.22 |
| SLEPDAI 1st trimester | 2 [0–2] | 2 [0–2] | 2 [0–2] | 2 [0.5–2] | 0.41 |
| SLEPDAI 2nd trimester | 2 [0–2] | 2 [0–2] | 2 [1–3] | 2 [0.5–2] | 0.74 |
| SLEPDAI 3rd trimester | 2 [0–2] | 0 [0–2] | 2 [2–4.5] | 2 [0.5–5.5] | <0.01 |
| SLEDAI < 6 months postpartum | 2 [0–4] | 2 [0–4] | 2 [0–5] | 4 [2–5.5] | 0.27 |
| Any flare before, during pregnancy, or postpartum | 44/144 (30.6) | 35/117 (29.9) | 5/14 (35.7) | 4/13 (30.8) | 0.94 |
| Severe flare before, during pregnancy, or postpartum | 5/144 (3.5) | 5/117 (4.3) | 0/14 (0) | 0/13 (0) | 1.00 |
| Mild/moderate flare before, during pregnancy, or postpartum* | 40/144 (27.8) | 31/117 (26.5) | 5/14 (35.7) | 4/13 (30.8) | 0.73 |
| <6 months before pregnancy | 9/144 (6.3) | 8/117 (6.8) | 0/14 (0) | 1/13 (7.7) | 0.67 |
| 1st trimester | 6/144 (4.2) | 5/117 (4.3) | 1/14 (7.1) | 0/13 (0) | 0.72 |
| 2nd trimester | 14/144 (9.7) | 9/117 (7.7) | 3/14 (21.4) | 2/13 (15.4) | 0.11 |
| 3rd trimester | 7/144 (4.9) | 6/117 (5.1) | 1/14 (7.1) | 0/13 (0) | 0.77 |
| <6 months postpartum | 20/144 (13.9) | 17/117 (14.5) | 2/14 (14.3) | 1/13 (7.7) | 0.91 |
| Medication started or dosage increased during pregnancy | | | | | |
| Prednisone | 25/144 (17) | 22/117 (19) | 2/14 (14) | 1/13 (8) | 0.77 |
| Prednisone | 25/144 (17) | 22/117 (19) | 2/14 (14) | 1/13 (8) | 0.77 |
| Azathioprine | 6/144 (4) | 5/117 (4) | 1/14 (7) | 0/13 (0) | 0.72 |
| Hydroxychloroquine | 4/144 (3) | 1/117 (1) | 0/14 (0) | 3/13 (23) | <0.01 |

Data depicted as median [interquartile range] or numbers (%). *A woman can flare multiple times before or during pregnancy or postpartum. SLE: systemic lupus erythematosus; aPL: antiphospholipid antibodies; APS: antiphospholipid syndrome; SLEDAI: SLE disease activity index; SLEPDAI: SLE disease activity index adjusted for pregnancy.

Table 3: Maternal and perinatal pregnancy complications in all study pregnancies.

| | Total | SLE-aPL | SLE + aPL | SLE + APS | $p$ value |
|---|---|---|---|---|---|
| *Maternal complications* | $N = 144$ | $N = 117$ | $N = 14$ | $N = 13$ | |
| Mild HD | 21/144 (14.6) | 18/117 (15.4) | 1/14 (7.1) | 2/13 (15.4) | 0.82 |
| Severe HD | 26/144 (18.1) | 19/117 (16.2) | 3/14 (21.4) | 4/13 (30.8) | 0.32 |
| Preeclampsia | 24/140 (17.1) | 18/113 (15.9) | 3/14 (21.4) | 3/13 (23.1) | 0.82 |
| Onset preeclampsia < 34 weeks | 8/24 (33.3) | 7/18 (38.9) | 1/3 (33.3) | 0 (0) | 1.00 |
| Eclampsia | 1/139 (0.7) | 1/112 (0.9) | 0/14 (0) | 0/13 (0) | 1.00 |
| HELLP syndrome | 7/144 (4.9) | 3/117 (2.6) | 1/14 (7.1) | 3/13 (23.1) | <0.01 |
| *Perinatal complications** | $N = 147$ | $N = 119$ | $N = 15$ | $N = 13$ | |
| IUFD | 6/147 (4.1) | 6/119 (5.0) | 0/15 (0) | 0/13 (0) | 1.00 |
| Preterm birth (<37 weeks) | 48/147 (32.7) | 40/119 (33.6) | 4/15 (26.7) | 4/13 (30.8) | 0.95 |
| SGA infant | 21/142 (14.8) | 18/115 (15.7) | 2/15 (13.3) | 1/12 (8.3) | 0.77 |
| Neonatal lupus | 2/147 (1.4) | 2/119 (1.7) | 0/15 (0) | 0/13 (0) | 1.00 |

Data depicted as numbers (%). *There were three twin pregnancies. SLE: systemic lupus erythematosus; aPL: antiphospholipid antibodies; APS: antiphospholipid syndrome; mild HD: hypertensive disorders of pregnancy including pregnancy induced hypertension; severe HD: hypertensive disorders of pregnancy including preeclampsia, eclampsia, and HELLP (hemolysis, elevated liver enzyme, and low platelet count syndrome); IUFD: intrauterine fetal death; SGA: small-for-gestational age (birth weight < p10).

occurred either in none of the pregnancies in the reproductive study period or in all studied pregnancies.

*3.4. Live Births.* The mean number of live births was $1.7 \pm 0.8$ standard deviation (SD) per patient during the studied reproductive period. The mean number of pregnancies per patient was $2.4 \pm 1.4$ SD (including miscarriages), and miscarriage rate was 14% with a mean of $0.33 \pm 0.7$ SD per woman during the studied reproductive period.

## 4. Discussion

In this study, we investigated the disease activity and maternal and perinatal complications of ongoing pregnancies

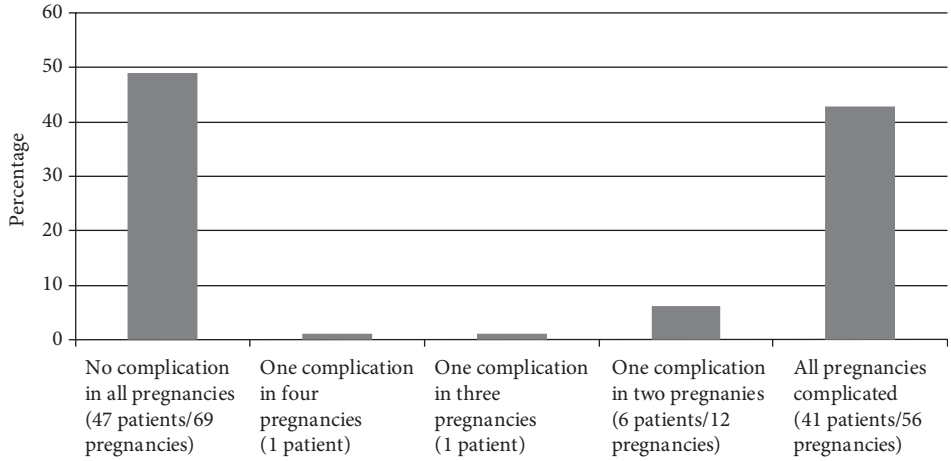


Figure 1: Percentage of pregnancy complications during the studied reproductive period. Complications included presence of any of the following: severe hypertensive disorders of pregnancy (including preeclampsia, eclampsia, and HELLP syndrome), placental abruption, preterm birth < 37 weeks, intrauterine fetal death, or small-for-gestational age infant.

(>16 weeks) in patients with SLE in the Netherlands in a real-life setting. Low disease activity was found before, during, and after pregnancy. Still, the incidence of maternal as well as perinatal complications was higher (especially preterm birth rate), compared to the general population, regardless of the overall low disease activity [30, 31]. The prevalences of severe HD, preterm birth, IUFD, and SGA infants were similar irrespective of antiphospholipid antibody status. One exception was HELLP syndrome, occurring more frequently in patients with SLE and APS. However, comparison between groups is difficult since the number of pregnancies within the SLE + aPL and the SLE + APS group were only 14 and 13, respectively. We also observed a similar complication rate in the first and consecutive pregnancies in contrast to the general population and a number of live births per women of 1.7. Both these findings are discussed later on.

In our patient population, only 6.3% experienced a mild flare before pregnancy, and no severe flares occurred. This is a reflection of planned parenthood facilitated by the close collaboration between rheumatologists and gynecologists in our centers. The (on average) low disease activity before pregnancy likely contributed to the low flare rate during pregnancy of around 20%, which is comparable with other studies reporting incidence rates between 10 and 33% [32–34]. The incidence of postpartum flares (<6 months) in our cohort was amounting to 15%. Importantly, patients with a flare during pregnancy were at greatest risk to develop a flare postpartum, and vice versa: 63.6% of flare postpartum occurred in patients with a flare before or during pregnancy. This finding calls for even more vigilance in the postpartum period especially in patients with increased disease activity during pregnancy.

The increase in use of HCQ after 2008 in the present study was neither associated with lower median disease activity scores during pregnancy nor associated with a reduced incidence of pregnancy complications. This finding is partly in line with the results of a recent retrospective study which demonstrated no difference in flare rates or maternal pregnancy complications such as severe HD and IUFD between patients treated with and without HCQ [32]. On the other hand, in the latter study, a reduction of mild HD (PIH) and preterm birth < 37 weeks was seen. Moreover, a prospective cohort study where HCQ was used in a similar number of pregnancies compared to the present study, and a small RCT suggested lower disease activity during pregnancy when HCQ was used [12, 18, 35]. This might be partly due to a different study population since we had an overwhelming majority of Caucasian women (84%) as opposed to Clowse et al. and Levy et al. (approximately 50–60%) [18, 35]. As SLE manifestations are known to be more severe in non-Caucasian women, the effect of HCQ may be greater in these patients. Also, adherence to medication could have played a role; we did not measure drug metabolites in the blood.

Maternal pregnancy complications occurred more often in our patients compared to those reported in the general population, including mild and severe forms of HD and preterm birth [30, 31]. In the general population, HD affect about 5–10% of all pregnancies and preterm birth occurs in less than 10% of all pregnancies in developed countries [30, 31]. The observed rate of 18.1% of HD in the present study is in line with other studies performed within patients with SLE [6, 36]. The percentage of patients who developed HELLP syndrome is low in our study with a significantly higher occurrence within the SLE + APS group compared to the other groups. Recently, Moroni et al. described maternal outcomes of prospectively followed pregnancies in women with a history of lupus nephritis and found a significant association between anti-beta-2 IgM antibody levels and preeclampsia/HELLP ($p = 0.048$) [11]. In our cohort, we only found an association with APS and HELLP. However, considering the low numbers of HELLP in the paper by Moroni et al. (2 out of 71 pregnancies = 2.6%) and our study (7 out of 144 pregnancies = 4.9%), we do not venture to interpret these findings.

We described that all patients with SLE + APS were treated with low-molecular-weight heparin during

pregnancy [29]. This is in agreement with the perceived increased occurrence of HELLP syndrome in patients with primary APS compared to mere aPL positivity in the literature [37, 38]. These findings suggest an important but not exclusive role for antiphospholipid antibodies in the development of HELLP syndrome.

The preterm birth rate was lower in the recent PROMISSE study, a prospective cohort study, compared to our study: 9% versus 24.3% < 36 weeks gestation, respectively [20]. This discrepancy might be explained by differences in the design of both studies and the definition of preterm (<37 weeks in our cohort versus <36 weeks in the PROMISSE study). In the PROMISSE study, patients with important comorbidity, for example, patients with diabetes mellitus or urinary protein–creatinine ratio greater than 1000 mg/g and patients using medium or high dosages of glucocorticoids were excluded. The preterm birth rate < 37 weeks found in our study is in line with the results of other studies [12, 39, 40].

We did not find a relevant increase in congenital abnormalities in our study. Congenital glaucoma, a rare congenital deformity with a prevalence of approximately 1 : 10,000 in Western population, occurred twice in our cohort, both children from the same mother suggesting a non-SLE-associated genetic influence, as was shown by Gencik et al. [41, 42].

To our knowledge, we compared for the first time the incidence of complications during the first and consecutive pregnancies in an SLE cohort. Shand et al. reported consecutive pregnancy outcomes within SLE patients using birth records without taking SLE disease characteristics into account [43]. We demonstrated that incidences of HD, preterm birth < 37 weeks, IUFD, and SGA are similar for consecutive pregnancies compared to the first pregnancy. This finding is not in line with observations in the general population, where nulliparity has been demonstrated as a risk factor for HD and multiparity reduces this risk, probably due to improvement of maternal-fetal immune adaptation in subsequent pregnancies [44]. We postulate that the maternal-fetal immune adaptation is different in patient with SLE. Patients with SLE should be informed about this finding in the preconceptional counseling.

Moreover, we examined pregnancy complications during the studied reproductive period. Remarkably, almost half of the patients did not develop any severe complication during all of their pregnancies and more than 50% developed at least one severe complication during any of their pregnancies. Although our finding may have been hampered by recall bias, it is a useful additional information in the preconceptional counseling.

The mean number of pregnancies per woman in our study was 2.4 and resulted in a mean number of live births of 1.7. This is similar to results of a case-control study in the late nineties with a mean number of pregnancies of 2.3 and mean number of live births of 1.8 [45]. Limitation of that case-control study is that the severity of SLE was not described. A review examining pregnancy loss (not further defined) showed a decrease of pregnancy loss between 1960 and 2000 [19]. The results of our study implicate that no further improvement in the number of pregnancies and number of live births has occurred over the last 15 years, although it is unknown if our population (with a history of nephritis and thrombosis in 39.6% and 16.0% of the pregnancies, resp.) is comparable with the population of Hardy et al. considering the information given in the publication [45].

The strength of the present study is that we did not use exclusion criteria with respect to disease activity, comorbidity, medication use, and twin pregnancies which enables us to present pregnancy outcomes of a complete SLE population reflecting real-life setting. Furthermore, by including all pregnancies per woman during a 16-year period of time, we were able to examine pregnancy complications between the first and consecutive pregnancies which, to our knowledge, have not been described before. A weakness of our study is that the majority of the population consisted of SLE patients without aPL which limits optimal comparison of women with aPL or APS.

## 5. Conclusions

In our cohort of 96 patients and 144 pregnancies, we made several observations: the incidence of maternal and perinatal complications is high compared to the reported rates in the general population, irrespective of antiphospholipid antibody status, despite low disease activity before, during, and after pregnancy. Furthermore, we compared for the first time disease activity and pregnancy outcomes in the first and consecutive pregnancies in patients with SLE and found that the incidence rates of HD, preterm birth < 37 weeks, IUFD, and SGA infants did not decrease in consecutive pregnancies, in contrast to the general population. Additionally, almost half of the women did not experience a severe complication including severe HD, placental abruption, preterm birth (<37 weeks), IUFD, and SGA infant in any pregnancy during their studied reproductive period (obstetric history and study). These observations could be of additional value in future counseling of SLE patients.

## Disclosure

The results presented in this article were presented in an oral presentation at the *Eular Annual European Congress of Rheumatology* in Madrid 2017.

## Conflicts of Interest

Jacob M. van Laar received honoraria from MSD, Pfizer, Roche, Eli Lilly, and BMS and research grants from MSD and Crescendo. Irene E. M. Bultink received speaker fees from Eli Lilly, MSD, Amgen, UCB, and Roche. Johanna I. P. de Vries received research grants from Pfizer. The other authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## Authors' Contributions

Sylvia J. Kroese and Carolien N. H. Abheiden are joint first authors.

## Acknowledgments

The study was supported by a single grant from the "Nationale Vereniging LE Patiënten (NVLE)," a Dutch patient organization.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 8596181, 6 pages
https://doi.org/10.1155/2017/8596181

*Clinical Study*

# Impacts of Anti-dsDNA Antibody on In Vitro Fertilization-Embryo Transfer and Frozen-Thawed Embryo Transfer

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Received 18 January 2017; Accepted 27 March 2017; Published 30 April 2017

Academic Editor: Jacek Tabarkiewicz



Our purpose is to explore whether anti-dsDNA antibody, which was demonstrated to enter living cells and induced apoptosis, could adversely affect reproductive outcomes. A total of 259 women receiving the in vitro fertilization-embryo transfer (IVF) cycle were enrolled in this study, including 52 women with positive ANA and anti-dsDNA (ANA+/anti-dsDNA+ group), 86 women with positive ANA and negative anti-dsDNA (ANA+/anti-dsDNA− group), and 121 women with negative ANA and anti-dsDNA (ANA−/anti-dsDNA− group). 136 nonpregnant women among 259 patients in the IVF-ET cycle were enrolled in the hormone replacement therapy frozen-thawed embryo transfer (HRT-TET) cycle. We compared basic characters and IVF outcomes among three groups in fresh embryo transfer and frozen-thawed embryo transfer cycle, respectively. The number of retrieved oocytes, available embryos, and high-quality embryos in the ANA+/anti-dsDNA+ group was lower than those in the other two groups in the fresh embryo transfer cycle. The rates of fertilization, implantation, and clinical pregnancy in the ANA+/anti-dsDNA+ group were the lowest, while the early miscarriage rate was the highest in the ANA+/anti-dsDNA+ group both in the fresh embryo transfer cycle and in the frozen-thawed embryo transfer cycle. Our data suggested that anti-dsDNA antibody may be the essential marker for defective oocytes or embryos in infertile women with any type of ANA.

## 1. Introduction

Antinuclear antibodies (ANAs) were related to infertility, decline of oocyte quality, impairment of embryo development, recurrent spontaneous abortion, and IVF failure [1–4]. ANAs were a large group of autoantibodies targeting the entire cell including DNA, RNA, proteins, and/or their complexes. It is unknown which kinds of ANA were involved in poor reproductive outcomes. As a serological marker for diagnosis of systemic lupus erythematosus (SLE), anti-dsDNA antibody played a crucial role in the pathogenesis of lupus nephritis [5, 6]. There were increasing literature which showed that anti-DNA antibody could penetrate into living cells and interact with their intracellular target [7–13]. These studies from several laboratories contradicted prevailing immunologic dogma that cell interiors were inaccessible to antibodies. Anti-dsDNA antibody could be detected in cellular internal and deposit in the kidney and other organs when anti-dsDNA antibody was administrated into nonautoimmune mice in vivo trial [7, 14]. Similar findings were observed after coculture anti-dsDNA antibody with cells in vitro trail. Studies showed that anti-dsDNA antibodies were cytotoxic to cells and induced apoptosis [15–19]. It is plausible that anti-dsDNA antibody could lead to abnormal development of oocyte and embryo. Thus, the aim of this present study was to explore the clinical significance of anti-dsDNA antibody in oocyte, fertilization, and embryo implantation after IVF-ET and HRT-TET.

## 2. Materials and Methods

*2.1. Patients.* The first part of this study was to investigate influences of anti-dsDNA on IVF-ET cycle. According to the inclusion criteria, a total of 259 women who presented to the IVF program at the Reproductive Medicine Center, The First Affiliated Hospital of Sun Yat-sen University, from December 2013 to May 2016 were recruited. Recruitment criteria were age < 38 years, basal FSH < 10 IU/l, antral follicle count between 6 and 15, no prior history of ovarian surgery, and no prior history of chemotherapy. The main indications for the detection of ANA and anti-dsDNA included IVF failure (≥2 cycles), recurrent IUI failure (≥3 cycles), and history of spontaneous abortion. For all patients, the anti-dsDNA and ANA were tested before the IVF program by the hospital's clinical laboratory. Infertility diagnoses for all patients were as follows: tubal disease in 66 patients, male infertility in 75 couples, combined male and tubal infertility in 53 couples, endometriosis in 22 patients, ovulatory disorders and other diagnoses in 23 patients, and unexplained infertility in 20 couples. Patients with autoimmune diseases or clinical presentations of autoimmune diseases, such as systemic lupus erythematosus, antiphospholipid syndrome, Sjogren's syndrome, scleroderma, and autoimmune thyroiditis, as well as those positive for any other autoantibodies including anticardiolipin antibody, antithyroid antibody, lupus anticoagulant, and rheumatoid factor were excluded from this study. Patients were divided into three groups according to the antibodies profile. A total of 259 women receiving the in vitro fertilization- (IVF-) embryo transfer cycle were enrolled in this study, including 52 women with positive ANA and anti-dsDNA (ANA+/anti-dsDNA+ group), 86 women with positive ANA and negative anti-dsDNA (ANA+/anti-dsDNA− group), and 121 women with negative ANA and anti-dsDNA (ANA−/anti-dsDNA− group). Impacts of anti-dsDNA on the frozen-thawed embryo transfer cycle were explored in the second part. 136 nonpregnant women among 259 patients in the IVF-ET cycle were enrolled, 32 women with positive ANA and anti-dsDNA (ANA+/anti-dsDNA+ group), 48 women with positive ANA and negative anti-dsDNA (ANA+/anti-dsDNA− group), and 56 women with negative ANA and anti-dsDNA (ANA−/anti-dsDNA− group) were included. All patients enrolled in this study were notified about the study, and all of them signed informed consent forms for publishing. The Medical Ethics Committee of The First Affiliated Hospital of Sun Yat-sen University approved this study.

*2.2. IVF Program.* Long-term pituitary downregulation was performed in all patients. In brief, a long-acting GnRH agonist (0.8 mg, 1.0 mg, or 1.3 mg) was administered subcutaneous in the midluteal phase. When complete pituitary downregulation was observed, gonadotropin was given for controlled ovarian hyperstimulation; the dose of gonadotropin was chosen according to the patient's age, antral follicle count, and basal FSH level. Human chorionic gonadotropin (5000–10,000 IU) was injected IM when at least two follicles > 18 mm or more than two follicles > 17 mm in diameter were present. Oocytes were collected approximately 36 hours after hCG injection and subjected to IVF. At most, three embryos were transferred into the uterus on the third day after oocyte collection. In our center, embryo quality is mainly assessed by a day-3 embryo grading system; a good quality embryo is defined as six to eight even, equally sized blastomeres with <20% fragmentation of blastomeres. High-quality embryo was given priority to choose for transfer, and high-quality embryos were transferred to all patients in the in vitro fertilization-embryo transfer cycle of this study. Fourteen days after ET, the urine and serum hCG levels were measured. Once urine and serum hCG examination showed positive results, the patients received ultrasonography 2 weeks later to determine the presence of clinical pregnancy.

*2.3. Endometrial Preparation in the Hormone Replacement Therapy Frozen-Thawed Embryo Transfer Cycle.* 136 nonpregnant patients among 259 patients in the IVF cycle began to take pentanoic acid estradiol orally at the third day of menstrual period after IVF-ET treatment for 3 months or above. Initial dose was selected on the basis of former endometrial conditions detected by ultrasound. Patients need to receive blood tests and ultrasonic inspections at regular intervals to adjust medical dosage according to the outcomes of endometrial thickness and serum level of sex hormones. When endometrial thickness reached 8 mm or above and serum level of estradiol was higher than 100 pg/ml, progesterone (40 mg/d) was intramuscularly injected to translate endometrium into the secretory phase. After the third day, embryos were transplanted at the fourth day of progesterone injection. At most, 3 embryos were allowed to transplant into the uterine cavity. Progesterone supplementation by daily intramuscular injection of 40 mg per day was applied for all patients. Pregnancy was diagnosed by positive blood test for HCG at 14 days after the third day of embryo transfer. Clinical pregnancy was confirmed by the detection of the gestational sac with a fetal heartbeat by transvaginal ultrasound examination after positive HCG test for 2 weeks.

*2.4. ANA and Anti-dsDNA Detection.* All patients detected anti-dsDNA and ANA before the IVF program.

Serum ANAs were detected by the indirect immunofluorescence assay (IFA) on a slide with human epithelial HEp-2 cell line and liver tissue (monkey) substrate (EUROIMMUN, Lübeck, Germany) in dilution ratios of 1 : 100, 1 : 320, and 1 : 1000. ANAs would react with the antigens in the HEp-2 cell substrate, forming antigen antibody complexes bound to the cell nucleus. The slides were prepared following the manufacturer's recommendations and protocol and were evaluated under the fluorescence microscope using ×20 or ×40 objectives. The ANA test was considered positive when the characteristic fluorescent signal was detected in the tissue or cells, with a serum dilution ratio of 1 : 100 (EUROIMMUN). Anti-dsDNA was tested by the indirect immunofluorescence assay (IFA) on a slide with *Crithidia luciliae*. Anti-dsDNA was defined as positive only when fluorescence staining was detected in kinetoplasts of

*Crithidia luciliae*. All assays were performed and interpreted according to the manufacturer's protocol.

*2.5. Data Collection.* The basic clinical information was collected including the age, duration of infertility, basal level of FSH and LH, endometrial thickness on the day of HCG injection, and number of embryo transferred and data of outcomes of IVF: number of oocytes retrieved, available embryos, high-quality embryos, fertilization rate (2Pb or 2PN oocytes in each group/total retrieved oocytes in the IVF cycle), clinical pregnancy rate (patients detected with fetal heartbeat in each group/total patients in each group), implantation rate (embryos with fetal heartbeat in each group/total transferred embryos), and early miscarriage rate (patients with pregnancy loss within 12 weeks of clinical pregnancy in each group/total patients with clinical pregnancy).

*2.6. Statistics.* Statistical analysis was done with SPSS version 20.0 statistic software package. The Kruskal-Wallis test was performed to analyze differences in quantitative data among the three groups, and the Mann-Whitney $U$ test was used to analyze differences between any two groups if difference among the three groups was statistically significant. A value of $P < 0.05$ was considered to indicate statistical significance among the three groups, and a $P$ value of <0.0167 was used to indicate statistical significance between any two groups. A chi-squared test and partition of chi-squared test were used to compare qualitative data; likewise, $P < 0.05$ was considered to be statistically significant among the three groups, and $P < 0.0167$ was used to indicate statistical significance between two groups. Fisher's exact test was used as appropriate.

## 3. Results

*3.1. Background of Patients' Characteristics.* Either in the fresh embryo transfer cycle or the in frozen-thawed embryo transfer cycle did not exist marked differences in the age, AFC, duration of infertility, bFSH, and bLH among the three groups (Tables 1 and 3).

*3.2. Controlled Ovarian Stimulation and IVF-ET Outcome.* Statistical analysis showed there were no significant differences in the days of ovarian stimulation, total Gn dose, E2 level on the day of HCG treatment, and the number of transferred embryos among the three groups, while the number of retrieved oocytes, available embryos, and high-quality embryos were the lowest in the ANA+/anti-dsDNA+ group. Either difference of comparison of retrieved oocytes between the ANA+/anti-dsDNA+ group and the ANA+/anti-dsDNA − group ($P < 0.001$) or difference of comparison of retrieved oocytes between the ANA+/anti-dsDNA+ group and the ANA−/anti-dsDNA− group ($P < 0.001$) was statistically significant. There was no difference in retrieved oocytes between the ANA+/anti-dsDNA− group and the ANA −/anti-dsDNA− group ($P = 0.439$), which was conformed with our previous study outcome. Significant differences were found in the number of available embryos and high-quality embryos between any two groups. Significant difference was also found in fertilization rate by partition of chi-squared test between any two groups. Although the difference of implantation rate between the ANA +/anti-dsDNA+ group and the ANA+/anti-dsDNA− group ($P = 0.176$) was not statistically significant, either difference between the ANA+/anti-dsDNA+ group and the ANA −/anti-dsDNA− group($P = 0.001$) or difference between the ANA+/anti-dsDNA− group and the ANA−/anti-dsDNA− group ($P = 0.008$) was statistically significant. In addition, the differences of pregnancy rate between any two groups were statistically significant. The pregnancy rate was the highest in the ANA−/anti-dsDNA− group. The early miscarriage rate was not statistically analyzed because of the small sample size (Table 2).

*3.3. HRT-FET Outcomes among the Three Groups.* HRT-FET outcomes were the same with IVF-ET outcomes. Although the difference of implantation rate between the ANA +/anti-dsDNA+ group and the ANA+/anti-dsDNA− group ($P = 0.535$) was not statistically significant, either difference between the ANA+/anti-dsDNA+ group and the ANA −/anti-dsDNA− group($P = 0.008$) or difference between the ANA+/anti-dsDNA− group and the ANA−/anti-dsDNA− group ($P = 0.015$) was statistically significant. In addition, the differences of pregnancy rate between any two groups were statistically significant. The pregnancy rate was the highest in the ANA−/anti-dsDNA− group. The early miscarriage rate was not statistically analyzed because of the small sample size (Table 3).

## 4. Discussion

Many studies have investigated the association of ANA with adverse reproductive events including infertility, miscarriage, and implantation failure [1–4]. In our previous clinical study, we found rates of mature oocyte, normal fertilization, cleavage, high-quality embryo, implantation, and pregnancy in the ANA+ group which were inferior to the ANA− group notably [20]. Then, we collected follicular fluid and discarded the third-day embryos and found ANA existed in follicular fluid and human embryos using ELISA and immunofluorescence methods. The level of ANA in serum and follicular fluid have positive correlation. In addition, ANA positive in serum or follicular fluid was a risk factor for ANA positive in embryo. Patients detected ANA positive in discarded embryos achieved lower high-quality embryos and implantation rate, which may be a remainder that ANA also impaired high-quality transplanted embryos morphologically [21].

Antinuclear antibodies (ANAs) were a large group of autoantibodies targeting the entire cell including DNAs, RNAs, proteins, and/or their complexes. Anti-dsDNA antibody, one member of ANA, and serological marker for diagnosis of systemic lupus erythematosus could be eluted from the kidneys of patients with active nephritis, which suggested that the antibody might be important in induction of tissue damage. Anti-DNA and anti-RNP antibodies could actually enter living cells and interact with their intracellular target which was firstly reported by Alarcon-Segovia and colleagues in 1978 [9]. Many subsequent studies have confirmed this observation [10–13]. Following administration

Table 1: General characteristics among the three groups in the IVF cycle.

| Variables | ANA+/anti-dsDNA+ group ($n = 52$) | ANA+/anti-dsDNA− group ($n = 86$) | ANA−/anti-dsDNA− group ($n = 121$) | $P$ value |
|---|---|---|---|---|
| Age (y) | 30.88 ± 4.29 | 31.41 ± 3.94 | 32.02 ± 3.73 | 0.262 |
| BMI | 20.93 ± 2.84 | 21.29 ± 2.79 | 21.40 ± 3.10 | 0.647 |
| AFC | 9.15 ± 1.35 | 9.12 ± 1.40 | 9.20 ± 1.16 | 0.825 |
| Duration of infertility (y) | 5.02 ± 2.79 | 4.85 ± 2.83 | 4.39 ± 3.02 | 0.093 |
| Basal FSH (IU/l) | 5.81 ± 1.51 | 6.02 ± 1.46 | 5.77 ± 1.44 | 0.312 |
| Basal LH (IU/l) | 3.98 ± 1.68 | 3.83 ± 1.62 | 4.03 ± 1.86 | 0.865 |
| Basal E2 (pg/ml) | 34.90 ± 12.21 | 34.36 ± 12.87 | 36.41 ± 12.01 | 0.416 |

$P < 0.05$ was considered to be statistically significant.

Table 2: Comparison of COS and IVF outcomes among the three groups.

| Variables | ANA+/anti-dsDNA+ group ($n = 52$) | ANA+/anti-dsDNA− group ($n = 86$) | ANA−/anti-dsDNA− group ($n = 121$) | $P$ value |
|---|---|---|---|---|
| Stimulation length (d) | 11.06 ± 1.75 | 11.07 ± 1.70 | 10.80 ± 1.98 | 0.663 |
| Total Gn dose (IU) | 2404.17 ± 797.50 | 2400.24 ± 784.28 | 2337.06 ± 846.77 | 0.634 |
| E2 level on HCG day (pg/ml) | 3087.08 ± 927.41 | 3086.66 ± 1000.67 | 3183.55 ± 1086.38 | 0.866 |
| Endometrial thickness on HCG day (mm) | 10.50 ± 1.61 | 10.85 ± 1.80 | 10.67 ± 1.11 | 0.568 |
| Number of retrieved oocytes | 9.81 ± 1.19[A,C] | 12.52 ± 3.50 | 12.89 ± 3.24 | <0.001 |
| Fertilization rate | 51.0% (260/510) [A,B,C] | 57.9% (624/1077) | 69.0% (1076/1560) | <0.001 |
| Number of embryo transferred | 2.04 ± 0.19 | 2.01 ± 0.11 | 2.02 ± 0.29 | 0.779 |
| Available embryos | 4.15 ± 0.85[A,B,C] | 5.24 ± 1.23 | 5.81 ± 0.91 | <0.001 |
| High-quality embryos | 2.13 ± 0.34[A,B,C] | 2.99 ± 0.83 | 3.36 ± 0.79 | <0.001 |
| Implantation rate | 9.4% (10/106) [B,C] | 15.0% (26/173) | 25.8% (63/244) | <0.001 |
| Clinical pregnancy rate | 11.5% (6/52) [A,B,C] | 30.2% (26/86) | 47.1% (57/121) | <0.001 |
| Early miscarriage rate | 100% (6/6) | 38.5% (10/26) | 22.8% (13/57) | — |

[A,B,C]$P < 0.05$ was considered to be statistically significant among the three groups. $P < 0.0167$ was considered to be statistically significant between any two groups. $P < 0.0167$ versus those of the groups ANA+/anti-dsDNA+ and ANA+/anti-dsDNA−. $P < 0.0167$ versus those of the groups ANA+/anti-dsDNA− and ANA−/anti-dsDNA−. $P < 0.0167$ versus those of the groups ANA+/anti-dsDNA+ and ANA−/anti-dsDNA−.

Table 3: Basal characteristics and HRT-FET outcomes among the three groups.

| Variables | ANA+/anti-dsDNA+ group | ANA+/anti-dsDNA− group | ANA−/anti-dsDNA− group | $P$ |
|---|---|---|---|---|
| Patients | 32 | 48 | 56 | — |
| Age (yrs) | 30.78 ± 4.21 | 31.42 ± 3.79 | 32.43 ± 3.86 | 0.098 |
| BMI | 20.32 ± 2.38 | 21.15 ± 2.98 | 21.71 ± 3.54 | 0.176 |
| Duration of infertility (yrs) | 4.56 ± 1.64 | 4.38 ± 2.16 | 4.73 ± 0.92 | 0.093 |
| bFSH (IU/l) | 5.94 ± 1.68 | 5.95 ± 1.23 | 5.74 ± 1.70 | 0.546 |
| bLH (IU/l) | 3.98 ± 1.87 | 4.51 ± 1.72 | 3.85 ± 1.59 | 0.099 |
| bE2 (pg/ml) | 36.19 ± 12.62 | 32.65 ± 10.57 | 36.71 ± 12.29 | 0.186 |
| Endometrial thickness on progesterone day (mm) | 10.75 ± 1.65 | 10.65 ± 1.47 | 10.93 ± 1.02 | 0.482 |
| E2 level on progesterone day (pg/ml) | 160.66 ± 33.42 | 163.79 ± 29.06 | 158.70 ± 24.57 | 0.791 |
| Embryo transferred | 2.00 ± 0.25 | 2.04 ± 0.46 | 2.11 ± 0.31 | 0.382 |
| Implantation rate (%) | 10.9% (7/64)[B,C] | 14.3% (14/98) | 28.0% (33/118) | 0.006 |
| Clinical pregnancy rate (%) | 9.4% (3/32)[A,B,C] | 33.3% (16/48) | 57.1% (32/56) | <0.001 |
| Early miscarriage rate (%) | 100% (3/3) | 37.5% (6/16) | 21.9% (7/32) | — |

[A,B,C]$P < 0.05$ was considered to be statistically significant among the three groups. $P < 0.0167$ was considered to be statistically significant between any two groups. $P < 0.0167$ versus those of the groups ANA+/anti-dsDNA+ and ANA+/anti-dsDNA−. $P < 0.0167$ versus those of the groups ANA+/anti-dsDNA− and ANA−/anti-dsDNA−. $P < 0.0167$ versus those of the groups ANA+/anti-dsDNA+ and ANA−/anti-dsDNA−.

of anti-dsDNA antibody to nonautoimmune mice, anti-dsDNA antibody was detected in the nuclei of multiple cell types such as renal tubular cells, hepatocytes, neuronal cells, fibroblasts, and mononuclear cells. In the kidney, this was associated with glomerular hypercellularity and proteinuria. Nuclear localization was present after injection of F(ab) fragments of these anti-DNA antibodies, which indicated that localization occurred through the antigen binding region of the molecule and was FcR independent. Clinical symptoms of lupus nephritis appeared after injection of human or murine anti-dsDNA antibody into normal mice. Some studies demonstrated that anti-dsDNA could induce cell apoptosis after penetrated into cells [16–20]. Other studies also showed that intracellular penetration by anti-dsDNA antibody could upregulate mRNA expression of cytokines such as IL-1, IL-6, IL-8, and TGF-$\beta$ [22, 23].

Apoptosis, named programmed cell death, is essential to embryonic development, homeostasis, surveillance, and elimination of pathological changes. Its main features are chromosome condensation, chromosome breakage, reduced cell volume, and apoptosis body. The damage of DNA degradation by endonuclease is irreversible in the process of apoptosis [24]. Apoptosis is important for the development of oocyte and preimplantation embryo under physiological circumstances. Arrested development and death would be occurred if apoptosis degree beyond physiological range. Low quality of embryos were associated with apoptosis [25, 26]. High level of DNA fragment in oocytes was related to low fertility by TUNEL detection [27]. Nowadays, the method of assessment of embryonic development potential was embryo morphological score in major reproductive medicine centers. But the predictive value of this method was limited and subjective. Recent study proved that regulatory mechanism of apoptosis was critical to preimplantation embryo potential and apoptosis degree of embryo was one key factor of reflection of embryo quality [28]. Anti-dsDNA antibody induced rat mesangial cell apoptosis without affecting p53 and Fas gene expression [15]. Human neutrophil cell apoptosis also took place as coculture with anti-dsDNA derived from lupus mice [16]. Increased cleavage of DNA caused by anti-dsDNA illustrated that anti-dsDNA induced cell apoptosis [17]. According to these lines of evidence, which offered indirect proof that anti-dsDNA antibody in patients with infertility might infiltrate into the oocytes and embryo and induce apoptosis. That may be the reason for poor reproductive outcomes. Without doubt, whether higher rate of apoptosis existed in oocytes and embryos from anti-dsDNA antibody positive patients and whether anti-dsDNA antibody could be detected directly in oocytes and embryos from anti-dsDNA antibody positive patients need to be verified in the future experiments.

## 5. Conclusion

To date, there is little information on the clinical significance of anti-dsDNA for improvement of artificial reproductive technology (ART) outcome. The present study analyzed IVF and HRT-TET outcomes among the ANA+/anti-dsDNA+ group, the ANA+/anti-dsDNA− group, and the ANA−/anti-dsDNA− group and found that the number of oocytes retrieved, available embryos, high-quality embryos, rates of fertilization, and clinical pregnancy were the lowest in the ANA+/anti-dsDNA+ group. All differences were significant. Although the early miscarriage rate was not statistically analyzed because of the small sample size, we found that all pregnant patients suffered from abortion in the ANA +/anti-dsDNA+ group and that anti-dsDNA could induce oocyte and embryo apoptosis through entering living cell may be the mechanism for poor reproductive outcomes. These results may provide new approaches to understand autoantibody involvement in reproductive outcomes for anti-dsDNA positive women suffering from IVF failure.

## Ethical Approval

The Medical Ethics Committee of The First Affiliated Hospital of Sun Yat-sen University approved this study.

## Consent

Informed consent was obtained from all individual participants included in the study.

## Conflicts of Interest

All authors declare that they have no conflict of interest.

## Authors' Contributions

Jiao Fan participated in the data collection and management, data analysis, and manuscript writing. Yiping Zhong participated in the protocol development. Cuina Chen participated in the data collection.

## Acknowledgments

The study was supported by the Science and Technology Project of Guangdong Province (no. 2013B021800271), Science and Technology and Information Bureau of Guangzhou City (2014J4100161), and Population and Family Planning Commission of Guangdong Province (20132048).

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