Age at menarche, Tfh cells and subsequent reproductive performance: a follow-up and Mendelian Randomization study

Xiao-Hong Li[1], Mei-Yin Lu[1], Xu Chen[1], Dan-Ting Shen[1], Li-Yan Zhang[1], Xiao-Jun Shen[1], and Bin Liu[1]

[1]Affiliation not available

May 5, 2020

Abstract

Objective: The subsequent reproductive events induced by early age at menarche (AAM) are tightly linked to immune dysfunction. This study aimed to analyze whether immune functions mediate the association between AAM and subsequent reproductive performance. Design: a follow-up and Mendelian Randomization (MR) study. Setting: A women's and Children's hospital in Shenzhen, China. Population: Sixty-eight healthy reproductive Chinese women were admitted to pre-pregnancy physical examinations. Methods: Pre-pregnancy immune functions were analyzed by flow cytometry. Subsequent reproductive performance was studied by a 15-month follow up. The associations of immune functions with AAM or pregnancy status were analyzed. Lastly, the important association was further validated by a two-sample MR test using public data. Main Outcome Measures: Miscarriages, thyroid function at early pregnancy, and metabolic indexes at mid pregnancy. Results: We found that AAM was negatively associated with Tfh1/Tfh2 ratio (Spearman r=-0.283, P=0.019). Moreover, this pre-pregnancy index was positively associated with TSH at early pregnancy (Spearman r=0.363, P=0.032), a risk for spontaneous miscarriage (adjusted Relative risk (RR)=12.25, 95% confidence interval (CI)=1.72-87.46, P=0.013), and a shorter time to miscarriage (42 days vs. 115 days, log-rank P=0.038). Moreover, the MR test showed that 84 AAM-related SNPs can explain 19% of variance in PD1-naïve Tfh cells (Directionality P=4.28×10-10); LIN28B and chromosome 9p12 (LINC01505, TAL2 and TMEM38B) were their share genetic factors. Conclusion: The present study implied that Tfh cells might mediate the process of early AAM-induced reproductive events. Larger population studies and functional studies are warranted. Funding: None.

Introduction

Women experiencing early age at menarche (AAM) are very common(1)·(2)·(3). Early AAM increases the risk of onset of various diseases, including metabolic disorders(4), cardiovascular diseases(5, 6), autoimmune diseases(7, 8), gynecological diseases and tumors. Also, it is related to subsequent reproductive events in reproductive females, such as gestational diabetes mellitus (GDM)(9), ectopic pregnancies and spontaneous miscarriages(10). Early AAM may even affect the health of her offspring: Early menarche was associated with an increased prevalence of preterm birth (PTB)(11) and low birth weight (LBW) of newborns (12), while PTB and LBW are the risk factors for the rate of infant incidence and mortality.

Previous studies have shown that early AAM can lead to premature hormone exposure and obesity(13), thus risk for various diseases, such as metabolic disorders, cardiovascular diseases, gynecological diseases and tumors. Both of premature hormone exposure and pre-pregnancy obesity affect immune functions(14, 15). In recent years, some studies found that early AAM was associated immune-related conditions, such as systemic lupus erythematosus (SLE) and asthma: early AAM and hormone treatments were risk factors for the onset of SLE(8). Also, a new phenotype for asthma has been discovered among the female population—the earlier the AAM, the higher the clinical score for asthma(7). In a cross-sectional study, early AAM was found to be associated with the risk of subclinical hypothyroidism(16). It implied that early AAM accompanied by

early exposure to estrogen may trigger thyroid autoimmunity, which will further impair the gravidas and fetuses(17).

Moreover, the subsequent reproductive events associated with AAM are tightly linked to inflammation and immune functions. For example, maternal serum levels of circulating TNF-α, IL-6 and CRP are increased in GDM patients(18). Pelvic inflammation is identified as a risk factor of ectopic pregnancies(19). And uterine immunity are pivotal for successful implantation, and the aberration of immune function is related to spontaneous miscarriages(20). However, the logical connection between early AAM, immune functions and subsequent reproductive performance are scarcely studied. We hypothesized that immunity mediating the process of early AAM-induced pregnancy complications.

To validate this hypothesis, we firstly analyzed the association of pre-pregnancy immune functions with AAM and reproductive events by using flow cytometry in this study. Then we validated the causal relationship between AAM and immune function by two sample Mendelian Randomization (MR) test using public Genome-wide association (GWAS) data. Our reports are herein.

Methods and Materials

Study population. Data were analyzed from a single hospital in Shenzhen, a Southern Chinese city, in accordance with the relevant guidelines and regulations as set out by the approving body, the Ethics Committee, Shenzhen Baoan Women's and Children's Hospital, Jinan University (IRB:LLSC 2018-08-01). All the study participants donated a 5ml venous blood after gave a written informed consent. All participants' names and other identifying information were removed in all sections of this manuscript.

From September to November 2018, we recruited at random 72 fertile women undergoing pre-pregnancy physical examinations at our hospital, where the rate of response was >95%. The subjects were between the ages of 20 and 40 years old, and were all local Han Chinese and long-term residents in Shenzhen, China. With the exception of four persons who suffered from uterine fibroids, recurrent miscarriage, oligomenorrhea and cervicitis, 68 healthy women were included in this study. After obtaining the informed consent, information of the subjects such as age, height, weight, AAM, menstrual cycle and period, and reproductive history were collected by trained nurses through a questionnaire survey, and 5ml venous blood was collected for flow cytometry analyses. Indicators including peripheral blood red blood cell (RBC), white blood cell (WBC) and hemoglobin (Hb) were obtained from the results of physical examination.

Follow-up of pregnancy status. The pregnancy status of the subjects were followed-up by interview until December 2019, which was verified by serum human chorionic gonadotropin (HCG) and ultrasound, and the first day of last menstrual period (LMP) was recorded. Among of these subjects, there were 35 pregnancies. Each pregnant woman was scheduled to come back to our hospital for antepartum examinations according to our hospital routine methods. Moreover, each pregnancy early signs of miscarriage was timely detected and treated by our hospital, and 7 ones were maintained to birth (defined as threatened miscarriage); However another 8 ones were aborted (defined as spontaneous miscarriage, with the exception of chromosome abnormality, congenital deformity of the fetuses, and other secondary diseases of the gravidas and fetuses). The information on weight gain at early pregnancy, thyroid stimulating hormone (TSH), free T4 (FT4) and thyroperoxidase antibodies (TPO-ab) at early pregnancy, and blood pressure, fast glucose, triglyceride (TG), cholesterol (CHOL), and HbA1c during mid-trimester pregnancy was collected from medical records of antepartum examinations.

Flow cytometry analyses. Pre-pregnancy immune functions were tested according to our routine methods(21). Briefly, peripheral white cells were isolated from whole blood at pre-pregnancy physical examinations by adding FACS LYSING SOLUTION (BD Biosciences, USA). Then the precipitates were collected by centrifugation. After washed twice by phosphate-buffered saline (PBS, sigma, USA), and cells were labelled according to our published paper(21). Antibodies for APC-H7-conjugated anti-CD3; PE-Cy7-conjugated anti-CD4 and anti-CD28; FITC-conjugated anti-CD45RA; PE-conjugated anti-CD25; BV421-conjugated anti-CD56, anti-CD127; BV510-conjugated anti-CD8; Alexa Fluor 647-conjugated anti-CCR7 and anti-CXCR5; Alexa Fluor 488-conjugated anti-CD183, and BV421-conjugated anti- CD194 were purchased from

BD Biosciences.

Samples were run on a BD LSR Fortessa (BD Biosciences) at Shuangzhi Purui Medical Laboratory Co., Ltd. (China), and the data was analyzed using FlowJo 10.1 software (Tree Star Inc, USA).

Data Collection of public GWAS studies. The published GWAS data for AAM and Tfh cells were collected from UK Biobank (*http://www.nealelab.is/blog/2017/9/11/details-and-considerations-of-the-uk-biobank-gwas*) and TwinsUK(22), respectively.

Statistical analysis and public GWAS data analysis. The SPSS 25.0 statistical software (SAS Institute Inc., Chicago, IL, USA) was used to process and analyze the data. Data were presented as n , proportion, median and min/max. We first performed association analyses between immune functions and AAM by using Spearman correlation analyses. Manny-Whitney U tests were then used to compare immune cells between healthy pregnancies and threatened or spontaneous miscarriages. Spearman correlation analyses also were used to analyze the associations between Tfh cells and the various biological indexes during pregnancy. Lastly, we used the Kaplan-Meier estimate and Cox regression analyses to study the associations between Tfh cells and miscarriages. The relative risk (RR) and its 95% confidence interval (CI) were calculated. All tests were two sided, and the level of significance was set at 0.05. Statistical figures were produced by using GraphPad Prism8.0 (GraphPad Software, USA).

To test the genetic association of Tfh cells and AAM, MR-Base software (*http://www.mrbase.org/*) were used for two sample MR test according to the method as described(23). Briefly, inverse-variance weighted approach, weighted median method, MR-Egger method, and single mode and weighted mode method were used for MR tests as a web application. Then we performed a Causal direction test to measure MR causal estimates. Lastly, a leave-one-out analysis was also performed to assess whether the MR estimate is driven or biased by a single SNP.

Results

Patient characteristics. Sixty-eight healthy Chinese Han women for pre-pregnancy examination were included in this study, as described in our previous report(21). **Table S1** showed in summary the demographic characteristics of the 68 subjects. The mean age of the subjects was (29.9 ± 3.9) years, where 36 (52.9%) were younger than 30 years. The Body Mass Index (BMI) of 59 subjects (86.8%) was below 24 kg/m^2. The average AAM was (14 ± 1.6) years old, where 26 (38.2%) experienced AAM below the age of 14 years. The average menstrual cycle was 30 days, where 10 females (14.7%) had abnormal cycles, among of which two subjects (2.9%) had short menstrual cycles (less than 24 days) and 8 ones (11.8%) had prolonged menstruation (more than 35 days). In addition, 40 females (58.8%) had at least one gravidities, and 33 ones (48.5%) were parous. Twenty (29.4%) had one miscarriage, and nine (13.2%) have had more than two miscarriage.

There were 35 pregnancies after 15 months follow-up. Among of these subjects, the body weight of 10 ones (41.2%) increased more than 19.98 kg at early pregnancy. And the median of TSH, FT4 and TPO-ab of pregnancy subjects at early pregnancy were 1.9 mIU/ml, 10.4 pmol/L, and 32.1 IU/ml, respectively. During the mid-trimester pregnancy, the median of fast glucose, triglyceride, cholesterol , HbA1c, and systolic/diastolic blood pressure was 4.5 mmol/L, 2.1 mmol/L, 6.1 mmol/L, and 117/66 mmHg, respectively.

In these 35 gravidas, there were 20 healthy pregnancies, 7 threatened miscarriages and 8 spontaneous miscarriages. No chromosome abnormality and congenital deformity of the fetuses was found in these miscarriages.

Early AAM is associated with pre-pregnancy Tfh1/Tfh2 ratio. We analyzed the correlations between AAM and T cells, γδ T cells and their subsets according to the method as described(21). No significant correlations was found between AAM and CD3$^+$, double positive T (CD4$^+$CD8$^+$) and double negative (CD4$^-$CD8$^-$) T cells, T helper cells (CD4$^+$, also known as Th cells) and subsets Th1/2/17, and cytotoxic T cells (CD8+, also known as Tc cells) and subsets Tc1/2/17 (all P values >0.05). At the same time, no significant correlations were found between AAM and CD4/CD8 memory cells such as Naïve, central memory (CM), effect memory (EM), and EM CD45RA+ (EMRA). No significant correlations were found between AAM and γδ T cells either (all P values > 0.05).

Among T follicular helper cells ($CD3^+CD4^+CXCR5^+$, also known as Tfh cells), Tfh1 ($CD4^+CXCR5^+CXCR3^+CCR4^-$) were significantly negatively correlated with AAM (r =-0.273,P =0.027), wherever Tfh2 ($CD4^+CXCR5^+CXCR3^-CCR4^+$) were positively associated with AAM (Spearman r =0.342,P =0.005), as shown in **Figure 1B.** Further, the Tfh1/Tfh2 ratio also showed significantly negative correlated with AAM (Spearman$r$ =-0.283, P =0.019). There was no discernible correlation between Tfh17 ($CD4^+CXCR5^+CXCR3^-CCR4^-CCR6^+$) and AAM (Spearman r =-0.137, P =0.265).

Pre-pregnancy Tfh1/Tfh2 ratio is associated with TSH at early pregnancy. The pregnancy status of the subjects were followed-up by interview until December 2019. After a 15-month follow-up, there were 35 pregnancies, including 20 healthy pregnancies, 7 threatened miscarriages and 8 spontaneous miscarriages.

We analyzed the correlation between pre-pregnancy Tfh1/Tfh2 ratio and some physical examination indexes during pregnancy. We found that the ratios of Tfh1/Tfh2 at pre-pregnancy examinations were significantly positively associated with TSH at early pregnancy (Spearman$r$ =0.367, P =0.046, **Figure 2**).

However, there no significant associations between pre-pregnancy Tfh1/Tfh2 ratio and either of the following indexes: weight gain, FT4 and TPO-ab at early pregnancy; and BMI, blood pressure, fast glucose, triglyceride, cholesterol, and HbA1c during mid-trimester pregnancy (all$P$ values >0.05, as shown in **Table S2**).

Pre-pregnancy Tfh1/Tfh2 ratio was associated with an increased risk and a shorter time to spontaneous miscarriage. Consistent with previous reports(10), the average AAM of threatened miscarriages and spontaneous miscarriages was both early than healthy pregnancies (both$P$ values <0.05, **Table S3**).

Therefore, we analyzed the relationship between pre-pregnancy Tfh1/Tfh2 ratio and miscarriages. Firstly, Manny-Whitney U tests were used to compare immune cells between healthy pregnancies and threatened or spontaneous miscarriages. We found that Tfh1/Tfh2 ratio was higher in spontaneous miscarriages than the ratio in healthy pregnancies (median 0.288 vs. 0.179, Manny-Whitney U=-2.288, P =0.021, **Figure s1**), but not in threatened miscarriages.

We further studied the association of spontaneous miscarriage rates with the Tfh1/Tfh2 ratio and AAM during the study period in 35 gravidas. Rate of spontaneous miscarriages were analyzed by the Kaplan-Meier estimate and Cox regression analyses. The log-rank test was used to compare spontaneous miscarriages curves. We found that the women with AAM<14 years had a higher risk of spontaneous miscarriages (adjusted RR=17.28, 95% CI=2.04-146.75, P =0.009, **Table 1**), and a shorter spontaneous miscarriage time (45 days vs. 185 days, log-rank P =0.036, **Figure 3A**). The adjusted confounding factors included age, previous times of spontaneous abortion and BMI. Moreover, the women with the Tfh1/Tfh2 ratio[?]0.209 (median) also had a higher risk of spontaneous miscarriages (adjusted RR=12.25, 95% CI=1.72-87.46, P =0.013, **Table 1**) and a short time to the spontaneous miscarriage (42 days vs. 115 days, log-rank P =0.038,**Figure 3B**). Our results implied a possible logical connection with earlier AAM, higher pre-pregnancy Tfh1/Tfh2 ratio and spontaneous miscarriage.

However, no distinguishable association between mothers' menarche age and the gestational weeks and birth weight of newborns were found in this study.

Genetic factors of AAM contributes to Tfh cells. To test the causal relationship and shared genetic factors between Tfh cells and AAM, we performed two sample MR tests by using publicly available genetic summary data of Genome-wide association studies (GWAS) from 176008 subjects in UK Biobank (*http://www.nealelab.is/blog/2017/9/11/details-and-considerations-of-the-uk-biobank-gwas*) and 497 female participants from the UK Adult Twin Register (TwinsUK) (22) in MR-base(24).

At a GWAS threshold of statistical significance (P <5×10^{-8}; linkage disequilibriumR^2<0.1), we found that 84 AAM-related SNPs were significantly associated with $PD1^-$ naïve Tfh cells by Weighted median method (β=0.38±0.19, P =0.040,**Figure S2**) and by Weighted mode (β=0.62±0.31, P =0.045). Moreover, these SNPs can explain 19% of variance in $PD1^-$ Tfh naïve cells (Directionality$P$ =4.28×10^{-10}). However, there

were no significant causal relationship between AAM and two other types of naïve Tfh cells ($CCR4^-CXCR3^-$ and $CCR4^+CXCR3^+$), $CD4^+$Tfh cells and $CD8^+$ Tfh cells in MR-base (All *P*values > 0.05).

In MR-Base, there were another two AAM database: ReproGen consortium studies(25) and MRC-IEU consortium studies (*http://www.ewascatalog.org*). However, there were no significant association between AAM and Tfh cells by using GWAS Data from ReproGen and MRC-IEU consortium studies. These results might due to the small samples with outcomes in TwinsUK dataset, and the genetic background of TwinsUK might be more different to ReproGen and MRC-IEU consortium studies than to UK Biobank.

We further performed a leave-one-out analysis to assess whether the MR estimate is driven or biased by a single SNP. Using this method, we found that rs1516883 and rs1933801 were the major share genetic factors between AAM and $PD\text{-}1^-$ naïve Tfh cells (also shown as**Figure S2**). These two SNPs were located in chromosome region 9p12 (*LINC01505, TAL2* and *TMEM38B*) and near gene*LIN28B* respectively, both are reported AAM loci(25-28).

Discussion

Main Findings In this study, we found that higher Tfh1/Tfh2 ratio before pregnancy was seen in females with early AAM, and this increased Tfh1/Tfh2 ratio was significant with higher TSH level at early pregnancy. Moreover, the aberrant Tfh1/Tfh2 ratio was also related to a higher risk and a shorter time to spontaneous miscarriage. Lastly, The contribution of early AAM on hallmarks of Tfh cells was further validated by a two sample MR test.

Interpretation Tfh cells are differentiated from Naïve CD4 cells, and is required for B cell development(29). They interact with antigen-specific B cells in germinal center (GC), and promote somatic hypermutation, class switch recombination and affinity maturation of B cells(30). In addition, Tfh cells also facilitate the differentiation of GC B cells, transforming them into memory B cells and long-lived plasma cells(31). The hallmarks of the Tfh cells will lead to the uncontrolled activation of B cells, thus produce a large amount of antibodies(32). There were three subsets of Tfh cells in peripheral blood, Tfh1(CXCR3+CCR6-), Tfh2(CXCR3-CCR6-) and Tfh17 (CXCR3-CCR6+) cells[28,29]. Among of them, Tfh1 cells are major regulators of proinflammatory stress, and can strengthen the immune reactions(33); While Tfh2 cells induce the differentiation of naïve B cells into plasmablasts(34). Therefore, increase in Tfh1/Tfh2 ratio usually implicates an inflammation.

To the best of our knowledge, the present study revealed the association of early AAM with Tfh cells in women for the first time. The present study showed that genetic factors of AAM contributes to the variances of $PD1^-$ naïve Tfh cells. Compared to $PD1^+$ Tfh cells, $PD1^-$ naïve Tfh cells are less proliferative and produce less cytokines(35). It implied that early AAM will impair Tfh progenitor cells, thus aberrant Tfh1/Tfh2 ratio and unregulated humoral immunity. In shared genetic factors of AAM and Tfh cells, *LIN28B* encodes a key repressor of let-7 microRNA biogenesis and cell pluripotency(36). Transgenic *Lin28b* mice demonstrate altered pubertal growth(37). Moreover, LIN28B/let-7 pathway influences Naïve CD4 cells survival(38) and B cell development(39). let-7 is also aberrantly expressed in the embryonic chorion tissue of spontaneous abortion(40). Therefore, it is biological plausible that*LIN28B* involves the etiology of early AAM, aberrant Tfh cell level, and miscarriages. For chromosome region 9p12, it includes three AAM-related genes *LINC01505* (26)*, TAL2* (27) and*TMEM38B* (28). Among of them, *TAL2* is crucial for thymocyte development(41), and chromosome translocation involving this gene occurs in T cell acute lymphoblastic leukemia(42). Therefore, chromosome region 9p12 is linked to both AAM and immunity. Taken together above reports, our findings on the association of AAM and Tfh cells are supported by genetic evidences. Additionally, the longer hormone exposure and obesity, which are produced by early AAM(13), both are found to play pivotal roles in the function of Tfh cells in recent years(43-45). Thus, our findings on the relationship between early AAM and aberrant Tfh cells function are credible.

Another important finding of this study is the association between pre-pregnancy Tfh1/Tfh2 ratio and spontaneous miscarriage. Because humoral immunity plays a pivotal role in miscarriages(46), imbalance of pro-inflammatory and anti-inflammatory cytokines may lead to impairment of pregnancy(47). The relati-

onship between Tfh cells and spontaneous miscarriage is biological plausible. Different types of Tfh cells can secrete different antibodies: Tfh1 cells secrete IFN-γ, which can trigger to produce IgG2α, then cause abortion; Tfh2 secrete IL-4, which can trigger to produce IgG 1 and IgE, and was down-regulated in abortion women(48); Tfh17 secrete IL-17, which triggers IgA(49, 50). It can be concluded that the balance of Tfh1/Tfh2 is one of the important immune factors for abortion. When Tfh1 is dominant, the proportion of IFN-γ increases and IL-4 decreases, which will lead to abortion. Therefore, It is credible that Tfh cells, especially Tfh1 cells, mediates the process of early AAM related miscarriages, which was found in this study.

In this study, we also found that the pre-pregnancy ratio of Tfh1/Tfh2 was associated with TSH at early pregnancy. Thyroid diseases in women of childbearing age are mainly caused by autoimmune diseases(51). Autoimmune thyroid disease (AITD), including autoimmune hypothyroidism (Hashimoto's thyroiditis, HT) and autoimmune hyperthyroidism (Graves' disease, GD). It has been found that the percentage of Tfh cells is higher in HT patients and Tfh cells exist in the thyroid, suggesting that Tfh cells drive autoimmunity and participate in the pathogenesis(52). Abnormal immune microenvironment results in increased expression of CXCR5 in thyroid tissue, which leads to immune response of Tfh cells and the development of AITD(53). The disorder of thyroid function in pregnancy can cause hyperthyroidism or incomplete thyroid function. Women with hyperthyroidism during pregnancy are more likely to have obstetric complications such as miscarriage, premature birth, abruptio placentae, thyroid store, and heart failure or adverse neonatal outcomes such as prematurity, LBW, intrauterine growth restriction (IUGR) and still birth than normal women(17). Some studies have pointed out that even in healthy women without thyroid dysfunction, the risk of miscarriage, fetal death or neonatal death increases as TSH levels rise(54). Thus, we infer that the changes of the pre-pregnancy ratio of Tfh1/Tfh2immune function before pregnancy will affect the thyroid function during pregnancy, thus affecting the outcome of pregnancy.

Because early AAM also relates to many diseases, such as cardiovascular diseases(5), autoimmune diseases(7) and cancers(55), our study may hold potential to initiate new avenues of research.

Strengths and Limitations The most strength of the present study is using multiple analyses to show the mediation of Tfh cells in the process of early AAM-induced subsequent pregnancy events. First, we performed Spearman correlation analyses to study the relationship between AAM and Tfh cells at the pre-pregnancy examinations. Second, we used Manny-Whitney U tests, Spearman correlation analyses, Kaplan-Meier estimate and Cox regression analyses to investigate the association between AAM-related pregnancy indexes by a follow-up study. The pregnancy indexes included pregnancy status, thyroid function at early pregnancy, and metabolic indexes at mid pregnancy, gestational weeks and birth weight of newborns. Last but not the least, we validated the association between early AAM and aberrant function of Tfh cell by a two-sample MR test. Therefore, this preliminary work may provide a proof-of-concept for evaluating the role of Tfh cells in early AAM-related reproductive events.

The present study has some limitations: First, the pilot study was conducted in a small Chinese population, while the genetic data that support its findings were collected from two consortiums that were of European origin. The generalizability of our findings needs to be confirmed. Second, other productive events such as ectopic pregnancies cannot be analyzed because of the limited sample size. Third, many women have improved their awareness of pregnancy examination, such as blood glucose and blood pressure during pregnancy, which have been well controlled. Forth, there were only 497 females in TwinsUK. To avoid overestimate of locus-specific effects from GWAS data(56), the genetic contribution of Tfh cells to spontaneous miscarriages was not analyzed in this study.

Conclusion The present study found that early AAM may produce hallmarks of Tfh cells, then induce subsequent reproductive events, such as miscarriages and higher TSH levels. Our results may provide a new concept on the etiology of the AAM-related reproductive events. Larger population studies and functional studies are warranted.

Ethics approval

The present study was approved by the Ethics Committee of the Shenzhen Baoan Women's and Children's

Hospital, Jinan University for this study protocol (Date of approval: August 18th, 2018; reference number: LLSC 2018-08-01). Local hospital research and development approval was also obtained.

Acknowledgements

Guangdong Purui Biotechnology Co. Ltd. performed flow analyses.

Contribution to Authorship

BL designed the study. XL, ML and XC analyzed the data and wrote the paper. XL, ML, DS, LZ and XS performed the tests. XC made critical suggestions. BL approved the final version of the manuscript for publication.

Disclosure of Interests

The authors declare no competing interests.

Funding

This manuscript was not supported by any funds.

References

1. Anderson SE, Must A. Interpreting the continued decline in the average age at menarche: results from two nationally representative surveys of U.S. girls studied 10 years apart. J Pediatr. 2005;147(6):753-60.

2. Jaruratanasirikul S, Sriplung H. Secular trends of growth and pubertal maturation of school children in Southern Thailand. Ann Hum Biol. 2015;42(5):447-54.

3. Papadimitriou A. The Evolution of the Age at Menarche from Prehistorical to Modern Times. J Pediatr Adolesc Gynecol. 2016;29(6):527-30.

4. Kim Y, Je Y. Early Menarche and Risk of Metabolic Syndrome: A Systematic Review and Meta-Analysis. J Womens Health (Larchmt). 2019;28(1):77-86.

5. Ley SH, Li Y, Tobias DK, Manson JE, Rosner B, Hu FB, et al. Duration of Reproductive Life Span, Age at Menarche, and Age at Menopause Are Associated With Risk of Cardiovascular Disease in Women. J Am Heart Assoc. 2017;6(11).

6. Bubach S, De Mola CL, Hardy R, Dreyfus J, Santos AC, Horta BL. Early menarche and blood pressure in adulthood: systematic review and meta-analysis. J Public Health (Oxf). 2018;40(3):476-84.

7. Castro-Rodriguez JA. A new childhood asthma phenotype: obese with early menarche. Paediatr Respir Rev. 2016;18:85-9.

8. Costenbader KH, Feskanich D, Stampfer MJ, Karlson EW. Reproductive and menopausal factors and risk of systemic lupus erythematosus in women. Arthritis Rheum. 2007;56(4):1251-62.

9. Li H, Shen L, Song L, Liu B, Zheng X, Xu S, et al. Early age at menarche and gestational diabetes mellitus risk: Results from the Healthy Baby Cohort study. Diabetes Metab. 2017;43(3):248-52.

10. Sandler DP, Wilcox AJ, Horney LF. Age at menarche and subsequent reproductive events. Am J Epidemiol. 1984;119(5):765-74.

11. Li H, Song L, Shen L, Liu B, Zheng X, Zhang L, et al. Age at menarche and prevalence of preterm birth: Results from the Healthy Baby Cohort study. Sci Rep. 2017;7(1):12594.

12. Coall DA, Chisholm JS. Evolutionary perspectives on pregnancy: maternal age at menarche and infant birth weight. Soc Sci Med. 2003;57(10):1771-81.

13. Apter D, Reinila M, Vihko R. Some endocrine characteristics of early menarche, a risk factor for breast cancer, are preserved into adulthood. Int J Cancer. 1989;44(5):783-7.

14. Fernandez-Real JM, Pickup JC. Innate immunity, insulin resistance and type 2 diabetes. Diabetologia. 2012;55(2):273-8.

15. Veru F, Laplante DP, Luheshi G, King S. Prenatal maternal stress exposure and immune function in the offspring. Stress. 2014;17(2):133-48.

16. Kotopouli M, Stratigou T, Antonakos G, Christodoulatos GS, Karampela I, Dalamaga M. Early menarche is independently associated with subclinical hypothyroidism: a cross-sectional study. Horm Mol Biol Clin Investig. 2019;38(1).

17. Aggarawal N, Suri V, Singla R, Chopra S, Sikka P, Shah VN, et al. Pregnancy outcome in hyperthyroidism: a case control study. Gynecol Obstet Invest. 2014;77(2):94-9.

18. Pantham P, Aye IL, Powell TL. Inflammation in maternal obesity and gestational diabetes mellitus. Placenta. 2015;36(7):709-15.

19. Rana P, Kazmi I, Singh R, Afzal M, Al-Abbasi FA, Aseeri A, et al. Ectopic pregnancy: a review. Arch Gynecol Obstet. 2013;288(4):747-57.

20. Dekel N, Gnainsky Y, Granot I, Racicot K, Mor G. The role of inflammation for a successful implantation. Am J Reprod Immunol. 2014;72(2):141-7.

21. Varesano S, Zocchi MR, Poggi A. Zoledronate Triggers Vdelta2 T Cells to Destroy and Kill Spheroids of Colon Carcinoma: Quantitative Image Analysis of Three-Dimensional Cultures. Front Immunol. 2018;9:998.

22. Roederer M, Quaye L, Mangino M, Beddall MH, Mahnke Y, Chattopadhyay P, et al. The genetic architecture of the human immune system: a bioresource for autoimmunity and disease pathogenesis. Cell. 2015;161(2):387-403.

23. Liu J, Zhou H, Zhang Y, Huang Y, Fang W, Yang Y, et al. Docosapentaenoic acid and lung cancer risk: A Mendelian randomization study. Cancer Med. 2019;8(4):1817-25.

24. Hemani G, Zheng J, Elsworth B, Wade KH, Haberland V, Baird D, et al. The MR-Base platform supports systematic causal inference across the human phenome. eLife. 2018;7.

25. Day FR, Thompson DJ, Helgason H, Chasman DI, Finucane H, Sulem P, et al. Genomic analyses identify hundreds of variants associated with age at menarche and support a role for puberty timing in cancer risk. Nat Genet. 2017;49(6):834-41.

26. Shi J, Zhang B, Choi JY, Gao YT, Li H, Lu W, et al. Age at menarche and age at natural menopause in East Asian women: a genome-wide association study. Age (Dordr). 2016;38(5-6):513-23.

27. Perry JR, Stolk L, Franceschini N, Lunetta KL, Zhai G, McArdle PF, et al. Meta-analysis of genome-wide association data identifies two loci influencing age at menarche. Nat Genet. 2009;41(6):648-50.

28. Busch AS, Hagen CP, Assens M, Main KM, Almstrup K, Juul A. Differential Impact of Genetic Loci on Age at Thelarche and Menarche in Healthy Girls. J Clin Endocrinol Metab. 2018;103(1):228-34.

29. Andris F, Denanglaire S, Anciaux M, Hercor M, Hussein H, Leo O. The Transcription Factor c-Maf Promotes the Differentiation of Follicular Helper T Cells. Front Immunol. 2017;8:480.

30. Di Niro R, Lee SJ, Vander Heiden JA, Elsner RA, Trivedi N, Bannock JM, et al. Salmonella Infection Drives Promiscuous B Cell Activation Followed by Extrafollicular Affinity Maturation. Immunity. 2015;43(1):120-31.

31. Mitchison NA. T-cell-B-cell cooperation. Nat Rev Immunol. 2004;4(4):308-12.

32. Gensous N, Charrier M, Duluc D, Contin-Bordes C, Truchetet ME, Lazaro E, et al. T Follicular Helper Cells in Autoimmune Disorders. Front Immunol. 2018;9:1637.

33. Chen M, Guo Z, Ju W, Ryffel B, He X, Zheng SG. The development and function of follicular helper T cells in immune responses. Cell Mol Immunol. 2012;9(5):375-9.

34. Akiyama M, Yasuoka H, Yamaoka K, Suzuki K, Kaneko Y, Kondo H, et al. Enhanced IgG4 production by follicular helper 2 T cells and the involvement of follicular helper 1 T cells in the pathogenesis of IgG4-related disease. Arthritis Res Ther. 2016;18:167.

35. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, et al. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. Immunity. 2013;39(4):758-69.

36. Zhang J, Ratanasirintrawoot S, Chandrasekaran S, Wu Z, Ficarro SB, Yu C, et al. LIN28 Regulates Stem Cell Metabolism and Conversion to Primed Pluripotency. Cell Stem Cell. 2016;19(1):66-80.

37. Zhu H, Shah S, Shyh-Chang N, Shinoda G, Einhorn WS, Viswanathan SR, et al. Lin28a transgenic mice manifest size and puberty phenotypes identified in human genetic association studies. Nat Genet. 2010;42(7):626-30.

38. Pobezinskaya EL, Wells AC, Angelou CC, Fagerberg E, Aral E, Iverson E, et al. Survival of Naive T Cells Requires the Expression of Let-7 miRNAs. Front Immunol. 2019;10:955.

39. Kristiansen TA, Vanhee S, Yuan J. The influence of developmental timing on B cell diversity. Curr Opin Immunol. 2018;51:7-13.

40. He D, Zeng H, Chen J, Xiao L, Zhao Y, Liu N. H19 regulates trophoblastic spheroid adhesion by competitively binding to let-7. Reproduction. 2019.

41. Goldshtein A, Berger M. Friend or foe: can activating mutations in NOTCH1 contribute to a favorable treatment outcome in patients with T-ALL? Crit Rev Oncog. 2014;19(5):399-404.

42. Marculescu R, Le T, Simon P, Jaeger U, Nadel B. V(D)J-mediated translocations in lymphoid neoplasms: a functional assessment of genomic instability by cryptic sites. J Exp Med. 2002;195(1):85-98.

43. Kim DH, Park HJ, Park HS, Lee JU, Ko C, Gye MC, et al. Estrogen receptor alpha in T cells suppresses follicular helper T cell responses and prevents autoimmunity. Exp Mol Med. 2019;51(4):1-9.

44. Monteiro C, Kasahara TM, Castro JR, Sacramento PM, Hygino J, Centuriao N, et al. Pregnancy favors the expansion of circulating functional follicular helper T Cells. J Reprod Immunol. 2017;121:1-10.

45. Esteves de Oliveira E, de Castro ESFM, Cacador Ayupe M, Gomes Evangelista Ambrosio M, Passos de Souza V, Costa Macedo G, et al. Obesity affects peripheral lymphoid organs immune response in murine asthma model. Immunology. 2019;157(3):268-79.

46. Salle V, Schmidt J, Smail A, Maziere C, Conte MA, Brule A, et al. Antibodies directed against annexin A2 and obstetric morbidity. J Reprod Immunol. 2016;118:50-3.

47. Ehsani M, Mohammadnia-Afrouzi M, Mirzakhani M, Esmaeilzadeh S, Shahbazi M. Female Unexplained Infertility: A Disease with Imbalanced Adaptive Immunity. J Hum Reprod Sci. 2019;12(4):274-82.

48. Raghupathy R, Makhseed M, Azizieh F, Omu A, Gupta M, Farhat R. Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. Hum Reprod. 2000;15(3):713-8.

49. Jones AP, Kermode AG, Lucas RM, Carroll WM, Nolan D, Hart PH. Circulating immune cells in multiple sclerosis. Clin Exp Immunol. 2017;187(2):193-203.

50. Luckheeram RV, Zhou R, Verma AD, Xia B. CD4(+)T cells: differentiation and functions. Clin Dev Immunol. 2012;2012:925135.

51. Andersen SL. Frequency and outcomes of maternal thyroid function abnormalities in early pregnancy. Scand J Clin Lab Invest. 2019;79(1-2):99-107.

52. Liu Y, You R, Yu N, Gong Y, Qu C, Zhang Y, et al. Increased proportions of Tc17 cells and NK cells may be risk factors for disease progression in Hashimoto's thyroiditis. Int Immunopharmacol. 2016;40:332-8.

53. Li Q, Wang B, Mu K, Zhang JA. The pathogenesis of thyroid autoimmune diseases: New T lymphocytes - Cytokines circuits beyond the Th1-Th2 paradigm. J Cell Physiol. 2019;234(3):2204-16.

54. Benhadi N, Wiersinga WM, Reitsma JB, Vrijkotte TG, Bonsel GJ. Higher maternal TSH levels in pregnancy are associated with increased risk for miscarriage, fetal or neonatal death. Eur J Endocrinol. 2009;160(6):985-91.

55. Werneck AO, Coelho ESMJ, Padilha CS, Ronque ERV, Cyrino ES, Szwarcwald CL, et al. Age at menarche and cancer risk at adulthood. Ann Hum Biol. 2018;45(4):369-72.

56. Goring HH, Terwilliger JD, Blangero J. Large upward bias in estimation of locus-specific effects from genomewide scans. American journal of human genetics. 2001;69(6):1357-69.

Table 1.Analysis of Tfh1/Tfh2,AAM and risk of spontaneous miscarriage	**Table 1.Analysis of Tfh1/Tfh2,AAM and risk of spontaneous miscarriage n Individuals (%)**	**Table 1.Analysis of Tfh1/Tfh2,AAM and risk of spontaneous miscarriage n spontaneous miscarriages(%)**	**Table 1.Analysis of Tfh1/Tfh2,AAM and risk of spontaneous miscarriage Median time of spontaneous miscarriages (Days)**	**Table 1.Analysis of Tfh1/Tfh2,AAM and risk of spontaneous miscarriage Log-Rank *P* Value**	**Table 1.Analysis of Tfh1/Tfh2,AAM and risk of spontaneous miscarriage RR (95% CI)[a]**	**Table 1.Analysis of Tfh1/Tfh2,AAM and risk of spontaneous miscarriage Cox model *P* Value[a]**
Set	**35**	**8**	**46**			
AAM[?]14	23(65.7)	3(37.5)	185	0.036	1.00 (ref.)	
AAM<14	12(34.3)	5(62.5)	45		17.28 (2.04-146.75)	0.009
Tfh1/Tfh2<0.029	18(51.4)	2(25.0)	115	0.038	1.00 (ref.)	
Tfh1/Tfh2[?]0.209	17(48.6)	6(75.0)	42		12.25 (1.72-87.463)	0.013

The following abbreviations are used: RR, relative risk; ref., reference. [a] Cox regression analysis was adjusted for age, previous times of spontaneous abortion and BMI.	The following abbreviations are used: RR, relative risk; ref., reference. [a] Cox regression analysis was adjusted for age, previous times of spontaneous abortion and BMI.	The following abbreviations are used: RR, relative risk; ref., reference. [a] Cox regression analysis was adjusted for age, previous times of spontaneous abortion and BMI.	The following abbreviations are used: RR, relative risk; ref., reference. [a] Cox regression analysis was adjusted for age, previous times of spontaneous abortion and BMI.	The following abbreviations are used: RR, relative risk; ref., reference. [a] Cox regression analysis was adjusted for age, previous times of spontaneous abortion and BMI.	The following abbreviations are used: RR, relative risk; ref., reference. [a] Cox regression analysis was adjusted for age, previous times of spontaneous abortion and BMI.	The following abbreviations are used: RR, relative risk; ref., reference. [a] Cox regression analysis was adjusted for age, previous times of spontaneous abortion and BMI.

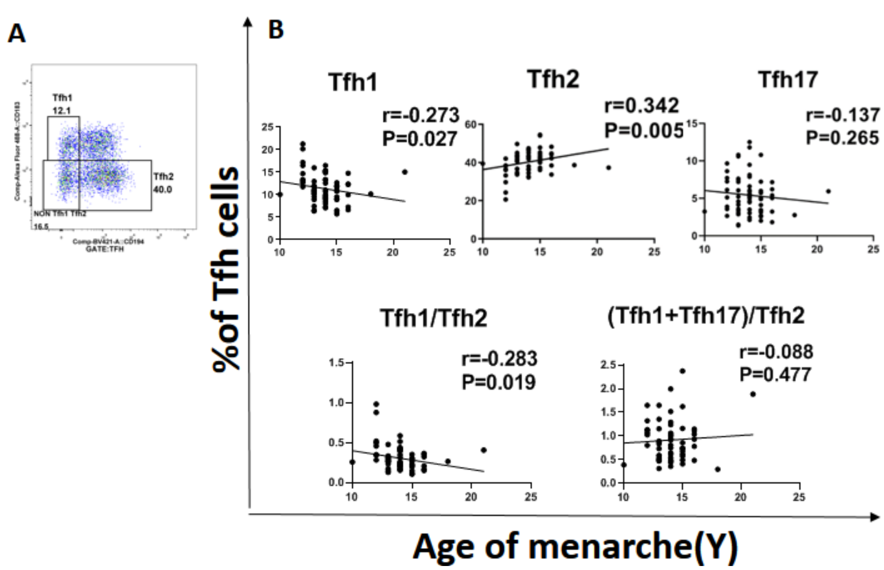
Figure 1. Tfh cells and the age at menarche. (A), Flow analysis of Tfh1, Tfh2 and Tfh17 cells. (B), Association between the subgroups of Tfh cells with the age at menarche.

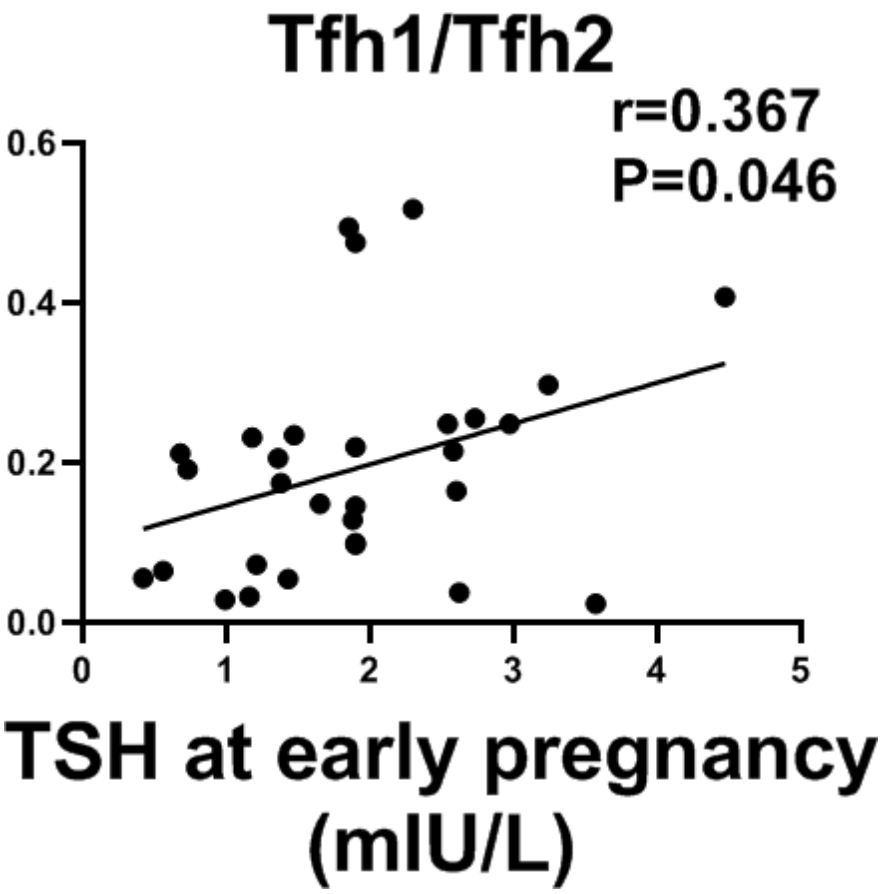
Figure 2. Correlation between pre-pregnancy Tfh1/Tfh2 and TSH at early pregnancy.

Figure 3. The rate of spontaneous miscarriage, plotted against time through 15 months of follow-up. (A), categorized by AAM. (B), categorized by ratio of Tfh1/Tfh2

Figure S1. Comparison of pre-pregnancy Tfh1/Tfh2 ratio in healthy pregnancies (H), threatened miscarriages (T) and spontaneous miscarriages (S) .

Figure S2. The causal relationship between AAM and Tfh cells: MR test between AAM and $PD1^-$ naïve Tfh cells, and the shared genetic loci between them: rs1933801 (*LIN28B*) and rs1516883 (Chromosome 9p12: *LINC01505, TAL* and *TMEM38B*).





Hosted file

Figure 3.tif available at https://authorea.com/users/303429/articles/433552-age-at-menarche-tfh-cells-and-subsequent-reproductive-performance-a-follow-up-and-mendelian-randomization-study