Plasma Neutrophil Extracellular Trap Remnant Levels Are Lower in Premenopausal Healthy Women Using Oral Contraceptive Pills

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that affects approximately 0.5-1% of the population with a 3-fold increased prevalence in females. The pathogenesis of RA involves inflammation and damage of joints, and can be characterized by the presence of RA-specific autoantibodies.

RA-associated inflammation involves neutrophil recruitment to the joints, and upon activation, the neutrophils undergo high levels of neutrophil extracellular trap (NET) formation in a process termed NETosis. NETosis is a process during which activated neutrophils decondense and externalize their cellular contents in net-like structures made of a DNA backbone. This process leads to the externalization of citrullinated proteins that can be the antigenic targets of RA-specific autoantibodies including anti-citrullinated protein antibodies (ACPA), which are clinically quantified using anti-cyclic citrullinated peptide (anti-CCP) assays. Due to the increased prevalence of RA in females, various studies have investigated a possible hormonal contribution to RA development. Moreover, while some factors such as tobacco use increase a person's risk of developing RA, other factors such as oral contraceptive pill (OCP) use have shown a protective effect. In addition, studies have found that OCP use was associated with a reduced risk of future development of RA. The exact mechanism by which these hormones impact RA pathogenesis has yet to be understood. This study was designed to address the question of what effect OCP use has on the process of NETosis and development of anti-CCP antibodies. The aims of the study include determining the prevalence of serum anti-CCP in women with varying OCP use as well as determining the systemic levels of NETosis in those same populations.

Methods

Samples: Utilizing samples from the Studies of the Etiologies of RA (SERA) cohort and participant level data, we included all premenopausal women without RA and without pre-RA (i.e. serum anti-CCP-IgG positive) who could be categorized into the following two groups: 1) current OCP users without prior use of other hormonal contraception, and 2) never/prior OCP users without current/prior use of other hormonal contraception. Groups were also stratified by whether participants did or did not have an FDR with RA.

Serum Analysis: Plasma samples were tested for NET remnants using immunofluorescence for cell free DNA (cfDNA, Quanti-iT PicoGreen dsDNA) and ELISA for calprotectin (Werfen). Serum was tested by ELISA for anti-CCP-IgA (research modification of CCP3.1, Werfen).

Statistical Analysis: Median levels of anti-CCP-IgA, NET remnants, age, and OCP duration were compared between groups by Wilcoxon rank sum test. Anti-CCP-IgA positivity and dichotomous clinical characteristics were compared between groups by Chi-squared testing. Linear regression models were used to compare NET remnant levels adjusted for clinical characteristics that could be potential confounders. Statistical analysis was performed using SPSS software.

This study was approved by the Colorado Multiple Institutional Review Board (COMIRB), protocol number 01-675.

Results & Discussion

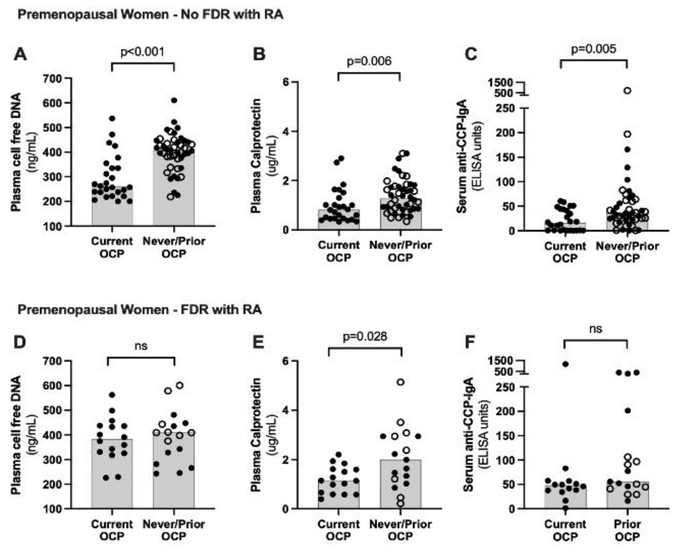
- FDRs were older, more likely smokers, and had higher anti-CCP-IgA levels.
- Median age of non-FDR group was 30 years compared to 36 years in the FDR group. History of ever smoking was higher in the FDR group compared to the non-FDR group.
- There were no significant differences history of pregnancy or presence of the shared epitope allele.
- While there was no difference in plasma cfDNA between FDRs and non-FDRs overall, anti-CCP-IgA was significantly lower in the non-FDR group ($p<0.01$) and there was a trend toward lower levels of calprotectin in non-FDRs ($p=0.07$).

Table 1. Clinical characteristics, plasma NET remnants and serum anti-CCP-IgA levels by group

	Non-FDRs (N=72)	FDRs (N=33)	p-value
Age	30 (24 - 38)	36 (27 - 46)	0.03
Ever smoker	3 (4%)	10 (31%)	<0.01
Ever pregnant	28 (39%)	17 (52%)	0.23
Current OCP use	26 (36%)	16 (49%)	0.23
OCP duration	6 (3 - 10)	11 (4 - 24)	0.08
≥1 Shared epitope allele*	22 (43%)	12 (44%)	0.91
cfDNA	383 (271 - 430)	398 (327 - 440)	0.25
Calprotectin	1.0 (0.7 - 1.6)	1.5 (0.9 - 2.2)	0.07
Anti-CCP-IgA	30 (11 - 51)	51 (38 - 91)	<0.01

Results are reported in median (IQR) or N (%)
*Shared epitope testing available in 51/72 non-FDRs and 27/33 FDRs

- In women without an FDR with RA, cfDNA, calprotectin and anti-CCP-IgA levels were significantly lower in current OCP users compared to never/prior OCP users.
- In women with an FDR with RA, calprotectin was significantly lower in current OCP users compared to never/prior ($p=0.028$), but cfDNA and anti-CCP-IgA did not differ between current and never/prior OCP users in those with a FDR with RA.
- There was no difference in cfDNA, calprotectin or anti-CCP-IgA levels between prior and never OCP users in the non-FDR or FDR groups ($p>0.05$ for all).



- In regression models adjusted for age, history of pregnancy and history of ever smoking, in the non-FDR group, cfDNA levels remained significantly inversely associated with current OCP use, while lower calprotectin maintained a trend toward an inverse association with current OCP use.
- In adjusted analyses in the FDR group, calprotectin levels remained significantly inversely associated with current OCP use. There was no correlation between duration of OCP use and cfDNA, calprotectin or anti-CCP-IgA levels ($p>0.05$).

Table 2. Results of multivariable linear regression for plasma NET remnant levels and current OCP use

	Non-FDRs (N=72)			FDRs (N=33)		
	Adjusted*			Adjusted*		
	Beta	95% CI	p-value	Beta	95% CI	p-value
cfDNA	-0.5	-142, -50	<0.01	-0.1	-95, 41	0.43
Calprotectin	-0.2	-0.7, 0.1	0.09	-0.5	-1.7, -0.3	<0.01

*Linear regression model adjusted for age, history of pregnancy and ever smoking

Many OCPs contain both estrogen and progesterone, and these hormones have been the topic of interest for many studies attempting to understand this relationship. While progesterone and androgens have been found to suppress the immune system, estrogens both stimulate and inhibit immune reactions. Various estrogens have been shown to increase T cell proliferation but they also can downregulate neutrophil activity. Progesterone decreases T cell proliferation and has been suggested to lead to the diminished immune response seen in pregnancy. Estrogen has also been shown to diminish T-cell function that can lead to increased osteoclast activation.

Conclusions

This study found lower levels of plasma NET remnants and anti-CCP antibody levels in premenopausal women without RA who were current OCP users. The finding was more pronounced in women without an FDR with RA. These findings suggest that reduced NETosis thereby reducing anti-CCP antibodies could be a potential mechanism by which OCPs contribute to a reduced risk of RA development in women. These findings support future prospective studies to better understand if NETosis modulation via OCP use can be utilized to reduce RA risk.

Future Directions

1. More investigation is necessary to understand the hormonal processes underlying OCP effects on NETosis and which components of OCPs play a role in various immune responses.
2. More investigation is needed to better understand the specific role of estrogen in these immunologic processes due to conflicting studies of this effect.
3. Further investigate if OCP use with current RA can reduce symptoms.