



Menstrual cycle-dependent alterations in glycosylation: a roadmap for defining biomarkers of favorable and unfavorable mucus

Monica Reynoso-Prieto¹ · Margaret Takeda¹ · Akraporn Prakobphol¹ · Dominika Seidman¹ · Sarah Averbach² · Susan Fisher¹ · Karen Smith-McCune¹

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Abstract

Objective To understand glycosylation of endocervical proteins at different times throughout the menstrual cycle in naturally cycling women and in women using hormonal or non-hormonal contraceptive methods, in order to characterize biochemical fingerprints of favorable and unfavorable cervical mucus.

Design Lectin/antibody-probed protein blot analysis of endocervical mucus samples collected onto ophthalmologic sponges (wicks) from two groups: a longitudinal cohort of naturally cycling women at three time points in their menstrual cycles (discovery cohort), and a cross-sectional cohort of women on hormonal or non-hormonal contraceptive methods (validation cohort).

Setting Participants were recruited from the San Francisco Bay Area from 2010 to 2016.

Patient(s) Women with regular cycles not using hormonal or intrauterine device (IUD) contraceptives were recruited for the longitudinal cohort ($n = 8$). Samples from women using levonorgestrel-containing combined oral contraceptives ($n = 16$), levonorgestrel containing IUDs ($n = 14$), copper IUDs ($n = 17$), depo-medroxyprogesterone acetate (DMPA) ($n = 15$), and controls ($n = 13$) were used for validation.

Intervention(s) None.

Main outcome measure(s) Detection of specific glycosylation patterns on lectin/antibody probed protein blots.

Result(s) Two lectins (*Lens culinaris* agglutinin and *Lycopersicon esculentum* [tomato lectin]), and the antibody MECA-79 demonstrated consistent cycle-dependent changes in protein binding. The glycan-binding patterns of the levonorgestrel-containing contraceptives were generally similar to each other and to those from women in the luteal phase. The DMPA samples showed slightly different binding patterns.

Conclusion(s) We identified molecular signatures of unfavorable mucus from women in the luteal phase and on hormonal contraceptives. Further characterization of these biomarkers may be useful in contraceptive development and in evaluation of infertility.

Keywords Glycosylation of endocervical proteins · Lectin blots · Cervical glycome · Cervical wicks · Endocervical fluid · Cervical glycans · Fertile window

Introduction

Currently available contraceptives do not meet the needs of many women. Based on a recent nationally representative survey, up to 40% of women are dissatisfied with their

contraceptive method [1]. Developing new contraceptive methods is a priority area for research and development [2]. In the National Survey of Family Growth from 2006 to 2010, 81.9% of sexually active women had used oral contraceptive pills, making it the most commonly used contraceptive method in the USA [3]. While the predominant mechanism of action of combined oral contraceptives is via inhibition of ovulation, other secondary mechanisms of action prevent pregnancy through effects on the cervix [4]. At the time of ovulation, cervical mucus increases in quantity, is thin and watery, and is permissive of sperm entry; these changes reverse after ovulation [5–7]. Exposure to synthetic progestins used in hormonal contraceptives and to high levels of

✉ Karen Smith-McCune
karen.mccune@ucsf.edu

¹ Department of Obstetrics, Gynecology and Reproductive Sciences, University of California San Francisco, San Francisco, CA, USA

² Department of Obstetrics, Gynecology and Reproductive Sciences, University of California San Diego, San Diego, CA, USA

endogenous progesterone in the luteal phase of the menstrual cycle results in cervical mucus that is thick and viscous, impeding sperm penetration, and therefore becoming unfavorable for conception [4, 7]. The biochemical properties underlying these alterations in cervical mucus structure are poorly understood; however, the amount and types of polysaccharide chains (glycans) added to proteins during the process of post-translational modification can result in dramatic alterations in protein molecular weight, solubility, viscosity, and biological function. Changes in mucus properties at the time of ovulation appear to depend largely upon changes in glycosylation rather than changes in protein expression [8]. Effects of endogenous and exogenous reproductive hormones on the glycosylation of cervical mucus proteins are not well described. A recent NIH-funded conference highlighted the need for the development of biomarkers of efficacy in order to advance the development of better contraceptives [9]. A better understanding of the biological composition of cervical mucus at different times in the menstrual cycle and in response to different hormonal contraceptives would be an important step towards reaching these goals.

Cervical mucus has traditionally been challenging to study due to its insolubility and complex biochemical structure, hindering a detailed analysis of its structure and its role in fertility and contraception [4–6]. The usual approach for sample collection has been to aspirate the mucus present in the endocervix using a pipette or syringe, or to collect the mucus in cervical cups. To advance the field, novel techniques for cervical mucus collection and evaluation are needed. We have found that endocervical fluid collected with ophthalmologic sponges (wicks) can serve as a rich source of glycoproteins secreted by endocervical cells. Our objective was to test the hypothesis that endocervical fluid collected onto cervical wicks can be used to measure changes in mucus glycans and would therefore be a suitable platform for biomarker discovery. We used glycan-specific reagents such as lectins and antibodies to study endocervical wick fluids from women at defined times in the menstrual cycle and on hormonal and non-hormonal contraceptives in order to identify reproducible glycosylation signatures that correlated with unfavorable mucus.

Methods

Study design

This study uses samples from two groups: a longitudinal cohort of cervical mucus samples at multiple time points within the menstrual cycle of women using no hormonal or intrauterine contraceptives (discovery cohort), and a cross-sectional cohort of samples from women using levonorgestrel-

containing combined oral contraceptives (COC), levonorgestrel-containing IUDs (LNG-IUD), copper IUDs (Cu-IUD), or depo-medroxyprogesterone acetate (DMPA), and as a control group, from the luteal phase of women using no hormonal or intrauterine contraceptives (validation cohort). The Institutional Review Board of the University of California San Francisco approved the study. Informed consent was obtained from all individual participants included in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Participant recruitment and eligibility

We recruited participants for the cross-sectional cohort from March 2010 to July 2012 and from Feb 2015 to August 2016 for other studies as described elsewhere [10, 11]. Samples for the longitudinal discovery cohort were collected specifically for this project from June to July 2016. Volunteers for both cohorts were recruited from the San Francisco Bay by ads on Craigslist, flyers in local clinics, and through social media, and respondents were pre-screened by telephone to confirm eligibility. Women aged 18–44 who had regular 27- to 30-day menstrual cycles and were either not at risk of pregnancy or currently using condoms or other non-hormonal methods for contraception were eligible. We excluded women if they were on systemic corticosteroids or immune modulating therapies, were currently or recently pregnancy or breastfeeding (within 3 months), or had a hysterectomy. Eligible candidates were screened for HIV by serology, for pregnancy by urine testing, and for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by self-collected vaginal swab.

For the longitudinal cohort, we collected endocervical wick samples at three points in a single menstrual cycle from each participant: (1) within 2–6 days after the start of menses, (2) approximately 1 week after visit 1, and (3) approximately 2–3 weeks after visit 1. We measured progesterone and estrogen levels at the last time point, and we used a progesterone level of > 2 ng/mL to indicate that the participant had ovulated.

For the cross-sectional study, samples from the control group (women not on hormonal or intrauterine contraceptives with serum progesterone > 2 ng/ml) or using DMPA or LNG-IUDs were collected as previously described [10, 11]. Women using COC had to be using a product containing the progestin levonorgestrel in a 28-day pack for at least 6 months and no more than 48 months; samples were collected between days 12 to 16 of the pill pack. Women using LNG- or Cu-IUDs were eligible if they had been using it for at least 6 months and no more than 48 months. Women using LNG- or Cu-IUDs were given ovulation detection kits (Procter and Gamble) and instructed to call when they ovulated; sample collection was

timed to be within 7–11 days after the test turned positive. We asked women on the LNG-IUD to test for ovulation for up to 2 months, and if there was no evidence of ovulation within that timeframe, we assumed that they were anovulatory and a sample was collected at a convenient time. We collected samples from 13 women for the control group, 16 women on COC, 14 women with LNG-IUDs, 17 women with Cu-IUDs, and 15 women on DMPA.

Sample collection and preparation

We collected endocervical fluid onto ophthalmic wicks (Beaver-Visitec) inserted into the cervical canal for 90 s. These were flash frozen and stored at – 80 °C until processing. Endocervical wicks were extracted using published methods [12] as previously performed in our laboratory [10, 11, 13]. Briefly, each wick was weighed and then extracted twice into ice cold extraction buffer (PBS, 0.25 M NaCl + 0.1 mg/ml aprotinin) [10]. Wicks were allowed to air dry for 24 h, then weighed again; the dry wick weight was subtracted from the initial wet weight to determine the net weight (i.e., volume) of fluid extracted from each wick. The protein concentration in each sample was determined using a Pierce™ BCA Protein Assay kit (ThermoFisher Scientific). Proteins were analyzed by denaturing polyacrylamide gel electrophoresis as follows: protein was loaded onto 1.5 mm 4–12% gradient gels (ThermoFisher Scientific) and run at 145 V for 90 min in 1X MES SDS running buffer (Thermo Fisher Scientific). Gel transfer was performed at 30 V for 90 min on to 0.45-µm nitrocellulose membrane paper (Thermo Fisher Scientific) in an XCell II™ Blot Module transfer chamber (Thermo Fisher Scientific) with 1X tris-glycine transfer buffer. After transfer, membranes were incubated in wash buffer (0.25 M Tris-HCl, 0.5 M NaCl, 0.5% Nonidet P-40) for 1 h and incubated with biotinylated lectins or antibody for 2 h at room temperature.

Membranes were rinsed and incubated in a 1:400 solution of streptavidin-HRP conjugate (Millipore Sigma) for 1 h. Bands were visualized by a colorimetric detection 3,3'-Diaminobenzidine (DAB+) liquid substrate chromogen system (Agilent).

Statistical methods/sample size

In this biomarker discovery project, we considered a meaningful signal on protein blots to be a loss or gain of a band at different times of the menstrual cycle in > 80% of our samples, with each participant serving as her own control for assessing gain/loss. For biomarker discovery, the use of ten samples for discovery is considered to have an 80% likelihood of reaching verification if a discrete number of biomarkers (fewer than 20) were tested [14]. We aimed for a sample size of approximately ten in the discovery and validation cohorts. No formal statistical analysis of the protein blots was performed.

Results

Table 1 shows the age, race, and length of contraceptive exposure of participants who contributed samples. For the longitudinal cohort, we enrolled 12 women but only had appropriate samples from 8 of them: One participant had a positive pregnancy test, one withdrew prior to completion, and two failed to ovulate (low progesterone).

Table 2 demonstrates the characteristics of the cervical mucus collected at each time point. The amount of protein recovered from the wicks ranged from approximately 0.5–6 mg per sample. The volume of fluid varied significantly across the cycle: the mean volume of wick fluid at time point 1 was 57.9 µL (standard deviation [SD] 46.1), time point 2 was 106.9 µL (SD 55.3), and time point 3 was 43 µL (SD 21)

Table 1 Demographics of study participants

Demographics	Longitudinal cohort <i>n</i> = 8	Cross-sectional cohort				
		Control (<i>n</i> = 13)	COC (<i>n</i> = 16)	LNG-IUD (<i>n</i> = 14)	Cu-IUD (<i>n</i> = 17)	DMPA <i>n</i> = 15
Age, median (min, max)	31 (21, 43)*	32 (21, 46)	25 (21, 33)	27 (20, 41)	28 (22, 33)	26 (21, 38)
Race, number (%)						
Asian	0	2 (15.4)	7 (43.8)	1 (7.1)	5 (29.4)	0
Black/African American	0	7 (53.9)	1 (6.25)	2 (14.3)	0	9 (60)
Unknown	0	0	2 (12.5)	1 (7.1)	1 (5.9)	0
White	8 (100)	4 (30.8)	6 (37.5)	10 (71.4)	11 (64.7)	6 (40)
Months of contraceptive exposure, median (min, max)	NA	NA	25 (9, 42)	20 (7, 36)	20 (6, 45)	15 (13, 33)

*Data missing for one participant

COC, combined oral contraceptives; LNG-IUD, levonorgestrel-containing IUDs; Cu-IUD, copper IUDs; DMPA, depo-medroxyprogesterone acetate; NA not applicable

Table 2 Properties of participant samples collected in the longitudinal cohort

	Time point 1			Time point 2			Time point 3							
	Total volume (μL)	Total Protein (μg)	Conc. (μg/μL)	Cycle day no.	Total volume (μL)	Total protein (μg)	Conc. (μg/μL)	Cycle day no.	Total volume (μL)	Total protein (μg)	Conc. (μg/μL)	Cycle day no.	Serum progesterone (ng/mL)	Serum estrogen (pg/mL)
CERV 01	22.3	1252	56.1	5	64.4	407.5	6.3	12	21.8	514	23.6	19	11.2	338.7
CERV 02	15.2	707.2	46.5	3	127.3	1022.3	8	10	34.1	579.7	17	17	7.1	522.6
CERV 05	125.7	4402	35	2	133.7	1173.6	8.8	11	28.1	683.4	24.3	18	7.7	298.9
CERV 07	39.5	2000	50.6	2	29.4	613.4	20.9	9	63.6	537.7	8.5	23	3.2	229.4
CERV 08	22.2	1173.6	52.9	4	82.6	952.3	11.5	13	38.8	417.2	10.8	18	3.1	327.4
CERV 09	84.3	2497.2	29.6	6	215.4	591	2.7	14	50.4	815	16.2	20	9.8	318.2
CERV 10	31	934.1	30.1	5	105.7	456.5	4.3	12	32.5	873.9	26.9	20	10.4	79.3
CERV 12	122.6	6056.1	49.4	4	96.5	1091	11.3	11	83.5	732.4	8.8	18	4.5	241.4

($p = 0.02$, one-way ANOVA). The protein concentration also varied significantly across the cycle: the mean concentration at time point 1 was $43.8 \mu\text{g}/\mu\text{L}$ (SD 10.6), time point 2 was $9.2 \mu\text{g}/\mu\text{L}$ (SD 5.6), and time point 3 was $17 \mu\text{g}/\mu\text{L}$ (SD 7.3) ($p = 0.00001$, one-way ANOVA).

Binding of *Lens culinaris* agglutinin (LCA) lectin to proteins from cervical mucus

The lectins used in this study and their target ligands are shown in Table 3. The majority of the lectins did not demonstrate any reproducible changes in binding patterns across the menstrual cycle (data not shown). However, we did see consistent cycle-dependent changes in binding of the lectin LCA. Figure 1a shows the LCA-binding patterns on samples from three representative participants. A band at 164 kilodaltons (kD) (outlined by dashed box) is present at time point 1 and absent at time point 3 in all samples. The band was also present, but fainter, at time point 2 in samples from participants 8 and 12. Figure 1a also shows a distinctive doublet of 100 kD and 91 kD at time point 1 (outlined by solid box). The upper band was present at all three time points, whereas the 91-kD band was present at time point 1, and absent at time point 3. When the LCA-binding patterns of samples from all 8 participants were compared to one another (data not shown), the 91-kD band was present in seven of the eight samples at time point 1, in 3 of the 8 at time point 2, and in 0 of 8 at time point 3, indicating that the ligand being detected is absent in luteal phase samples. We also confirmed these findings on pooled samples. Figure 1b shows the LCA-binding patterns in pools containing all samples from time points 1 (cycle days 2–6), 2 (cycle days 8–14), and 3 (cycle days 16–23). The bands at 164 kD (outlined by dashed box) and 91 kD (lower band in the solid box) are present at time point 1 and absent at time point 3, accurately reflecting what is seen on the individual samples.

To determine if the loss of LCA-bound bands at 164 kD and 91 kD was correlated with unfavorable mucus, we studied the binding patterns from samples collected under conditions in which the mucus would be unfavorable, i.e., women in the luteal phase (high progesterone) or on hormonal contraception. Figure 1c shows the LCA-binding pattern to pooled samples from the longitudinal cohort, as in Fig. 1b, and to pooled samples from control women in the luteal phase and from women on COC, LNG-IUD, Cu-IUD, or DMPA. The bands at 164 kD (outlined by dashed box) and 91 kD (lower band in the solid box) are clearly discernible only in time point 1 and not in any of the other pooled sample groups. These results confirm that the absence of these LCA-stained bands is correlated with conditions of high progestin levels and unfavorable cervical mucus.

Table 3 Summary of lectins and antibody used

Lectin/antibody	Carbohydrate ligand	Concentration (µg/mL)	Cycle-dependent pattern changes
<i>Aleuria aurantia</i> (AAL)	Fucα1-6/3GlcNAc	5	No
<i>Artocarpus integrifolia</i> (Jacalin)	Galβ1-3GalNAc, Galα1-6Gal	5	No
<i>Erythrina cristagalli</i> (ECA)	Galβ1-4GlcNAc	50	No
<i>Lens culinaris</i> agglutinin (LCA)	Fucα1-6GlcNAc-N-Asn containing N-linked oligosaccharides	12.5	Yes (Fig. 1)
<i>Lotus tetragonolobus</i> (LTL)	Fucα1-2Galβ1-4 (Fucα1-3) GlcNAc	5	No
<i>Lycopersicon esculentum</i> (tomato, TL)	(GlcNAcβ1-4) ₁₋₄	20	Yes (Fig. 2)
<i>Arachis hypogaea</i> (peanut, PNA)	Gal(β1,3)GlcNAc (T-antigen)	50	No
<i>Triticum vulgare</i> (wheat germ, WGA)	(GlcNAcβ1-4) ₂₋₅ Neu5Ac Manβ1-4GlcNAcβ1-4GlcNAc	12.5	No
MECA-79	L-Selectin ligand (SO ₃ -6GlcNAc)	5	Yes (Fig. 3)

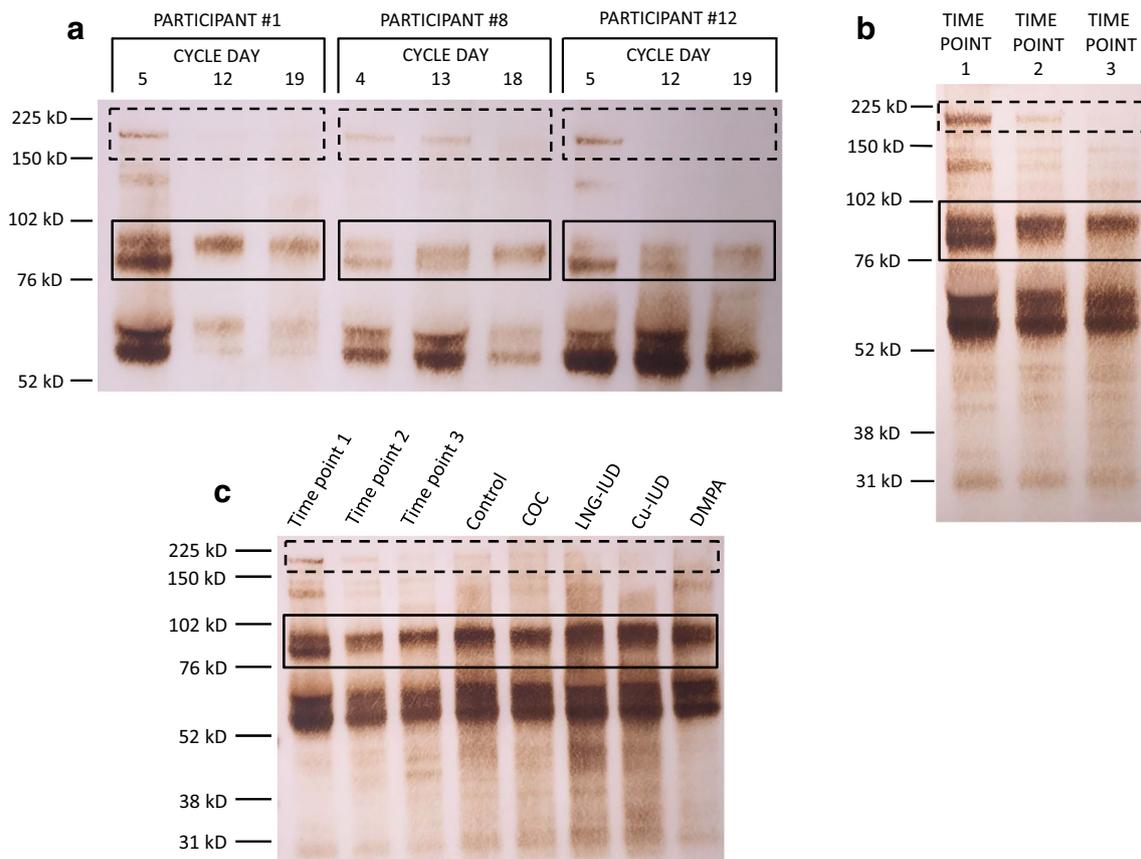


Fig. 1 Protein blots of cervical wick samples probed with the biotinylated lectin *Lens culinaris* agglutinin (LCA). Each lane was loaded with 10 µg of protein. **a** Samples from three participants at specific cycle days as shown. **b** Pooled samples from all eight participants at time point 1 (cycle days 2–6), time point 2 (cycle days 9–14), and time point 3 (cycle days 16–23). **c** Pooled samples from each time point as in Fig.

1b, and pooled samples from participants in the luteal phase on no hormonal or IUD contraceptive (control, *n* = 13), or using combined oral contraceptive (COC, *n* = 16), levonorgestrel-releasing intrauterine system (LNG-IUD, *n* = 14), copper IUD (Cu-IUD, *n* = 17), or Depo-medroxyprogesterone acetate (DMPA, *n* = 15)

Binding of *Lycopersicon esculentum* (tomato) lectin (TL) to proteins from cervical mucus

We observed consistent cycle-dependent patterns in TL binding in samples from the longitudinal cohort. Figure 2a shows TL-binding patterns in pooled samples from the three time points. A band at 164 kD (dashed box) is present in the pool at time point 1, faint at time point 2, and not detected at time point 3. There is also a complex of bands spanning from 20 to 33 kD (solid box). The intensity of staining of this protein complex increases across the menstrual cycle, with the darkest staining and clearest distinction of band patterns in the luteal phase (time point 3).

To determine if the loss of TL binding at 164 kD and increased binding at 20–33 kD was correlated with unfavorable mucus, we studied the binding patterns from samples collected under conditions in which the mucus would be unfavorable, i.e., women in the luteal phase (high progesterone) or on hormonal contraceptives. Figure 2b shows the TL binding pattern to pooled samples from the longitudinal cohort, as in Fig. 2a, and to pooled samples from the cross-sectional cohort of control women (luteal phase), and women on COC, LNG-IUD, Cu-IUD, or DMPA. The band at 164 kD (outlined by dashed box) is present at time point 1 and is not clearly discernible at time point 3 or any of the groups from the cross-sectional cohort. The complex at 20–33 kD (outlined by solid box) is similar in intensity to time point 3 (luteal

phase) in all groups from the cross-sectional cohort except for the DMPA group. Staining of this complex in the DMPA group is much fainter, more similar to the pattern seen in time points 1 and 2 of the longitudinal cohort, but with darker staining at the top of the complex than was seen in those samples.

Western blotting of MECA-79 to proteins from cervical mucus

We observed cycle-dependent changes in the binding of MECA-79, an antibody that recognizes a partial structure of carbohydrate moieties mediating L-selectin binding. Figure 3a shows MECA-79 binding patterns in pooled samples from the longitudinal cohort at the three time points. A band at 164 kD (outlined by dashed box) is present in the pooled samples at time point 1 and not detected at time points 2 or 3. A band at 85 kD decreases in intensity across the menstrual cycle. Bands at 50 and 44 kD (outlined by solid box) show varying intensities across the time points. The 50-kD band is faint at time point 1 and more intense at time points 2 and 3. The 44-kD band is only present in time points 2 and 3.

To determine if the observed changes are correlated with unfavorable mucus, we studied the binding patterns from samples collected under conditions in which the mucus would be unfavorable, as above. Figure 3b shows the MECA-79

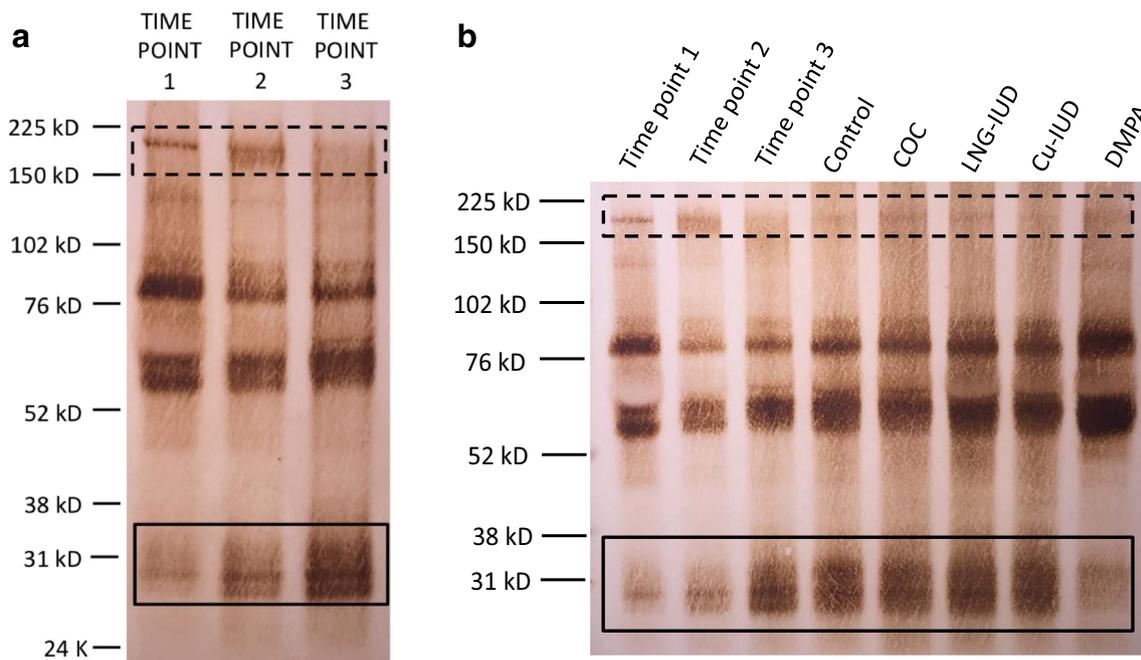


Fig. 2 Protein blots of cervical wick samples probed with biotinylated tomato lectin (*Lycopersicon esculentum*). Each lane was loaded with 20 μ g of protein. **a** Pooled samples from all eight participants at time point 1 (cycle days 2–6), time point 2 (cycle days 9–14), and time point 3 (cycle days 16–23). **b** Pooled samples from each time point as in Fig. 2a,

and pooled samples from participants in the luteal phase on no hormonal or IUD contraceptive (control, $n = 13$), or using combined oral contraceptive (COC, $n = 16$), levonorgestrel-releasing intrauterine system (LNG-IUD, $n = 14$), copper IUD (Cu-IUD, $n = 17$), or Depo-medroxyprogesterone acetate (DMPA, $n = 15$)

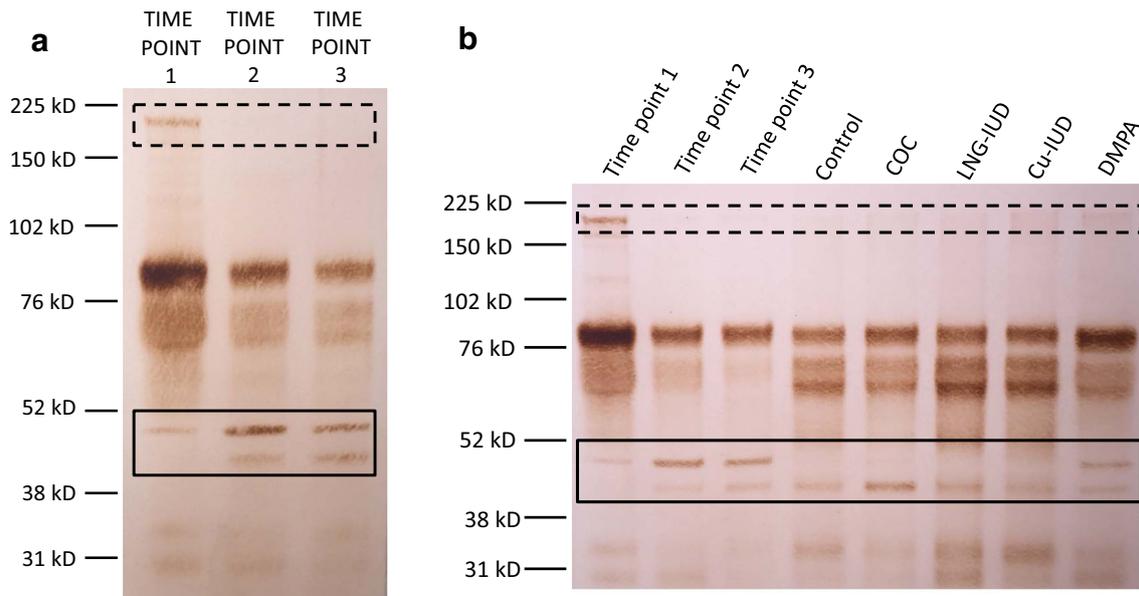


Fig. 3 Western blots of cervical wick samples probed with rat monoclonal IgM antibody MECA-79. Each lane was loaded with 10 μ g of protein. **a** Pooled samples from all eight participants at time point 1 (cycle days 2–6), time point 2 (cycle days 9–14), and time point 3 (cycle days 16–23). **b** Pooled samples from each time point as in Fig.

3a, and pooled samples from participants in the luteal phase on no hormonal or IUD contraceptive (control, $n = 13$), or using combined oral contraceptive (COC, $n = 16$), levonorgestrel-releasing intrauterine system (LNG-IUD, $n = 14$), copper IUD (Cu-IUD, $n = 17$), or Depo-medroxyprogesterone acetate (DMPA, $n = 15$)

binding pattern to pooled samples from the longitudinal cohort, as in Fig. 3a, and to pooled samples from the cross-sectional cohort of control women in the luteal phase, and women on COC, LNG-IUD, Cu-IUD, or DMPA. The band at 164 kD (outlined by dashed box) is present at time point 1 in the longitudinal samples but is not clearly discernible in any of the groups from the cross-sectional cohort. The 50-kD band that shows increased intensity at time points 2 and 3 is present only in the DMPA group in the cross-sectional samples. The 44-kD band is present in time points 2 and 3, controls, COC, LNG-IUD, Cu-IUD, and DMPA, confirming that its presence is correlated with conditions of unfavorable cervical mucus.

Discussion

In this study, we used samples of endocervical fluid collected onto ophthalmologic sponges (wicks) to examine glycoprotein composition of cervical mucus. Our results indicate that endocervical wick samples reflect physiologically relevant cycle-dependent changes in cervical mucus. The glycan-binding patterns of the levonorgestrel-containing contraceptives were generally similar to each other and to those from women in the luteal phase. The DMPA samples showed slightly different binding patterns.

Cervical wick samples have been validated as a useful source for studying endocervical proteins such as cytokines and chemokines [12], but to our knowledge, this is the first time they have been used for analysis of cervical mucus

glycans. The fluid volumes collected on the wicks were similar in scale to those collected by aspirating mucus from the endocervix [8]. In addition, wick fluid volume peaked in mid-cycle, consistent with the known increase in cervical mucus volume at the time of ovulation. Cervical wick samples are simple to collect and provide a relatively abundant source of soluble proteins, overcoming the difficulties of working with insoluble crosslinked mucins present in samples collected from menstrual cups or by aspiration of cervical mucus. Wick samples are thus tractable to biochemical analysis such as polyacrylamide gel electrophoresis, and their availability should accelerate research into biomarkers of fertility and contraception.

Lectins are a family of plant proteins that bind to specific polysaccharides and are thereby useful affinity reagents for identifying glycans [15]. MECA-79 is an antibody that recognizes a partial structure of carbohydrate moieties mediating L-selectin binding, which has been implicated in blastocyst binding to the endometrium [16]. Using these reagents, we demonstrated that endocervical wick samples contain a complex array of glycoproteins. In addition, we demonstrated reproducible cycle-dependent changes in expression of glycans detected by LCA, TL, and MECA-79, with some bands disappearing, and other bands appearing or becoming more intense from the early cycle time points to the luteal phase, a time of high progesterone levels. These specific affinity reagents were not meant to elucidate the functional significance of the patterns that we observed but rather were used to

identify changes in glycosylation that correlate with fertilization potential. These results validate our hypothesis that endocervical wicks provide a tractable source of material for identification of biomarkers of favorable versus unfavorable mucus. These results set the stage for comprehensive state-of-the-art proteomic and glycomic characterizations.

A previous study of O-glycosylation of proteins in cervical mucus revealed that the glycan profile before and after ovulation is dominated by sialylated oligosaccharides, and by neutral oligosaccharides at the time of ovulation [8], confirming the premise that the glycosylation of cervical mucus proteins varies with the menstrual cycle. Another study used lectin binding as a tool to study cervicovaginal fluid in samples from cycling and postmenopausal women and in women on hormonal contraceptives (COC, LNG-IUD, and DMPA) [17]. They collected fluid in menstrual cups and measured glycoprotein content with a plate-based enzyme-linked lectin assay (ELLA). They found that the levels of sialic acid binding were significantly lower in postmenopausal women, but otherwise did not vary by menstrual cycle or hormonal contraceptives.

Changes in carbohydrate-binding patterns across the menstrual cycle could be modulated by differences in side-chain glycosylation or sulfation in the Golgi apparatus, by removal of carbohydrate groups through cyclic expression of glycosidases, or by changes in expression of the protein scaffolds. A recent study looking at glycosidase enzymatic activity in cervicovaginal fluid from women at different phases of the menstrual cycle and on hormonal contraceptives found that the activity level of α -glucosidase was significantly lower in postmenopausal women than in the other groups, but there were no differences between follicular or luteal phase samples or contraceptive groups [17]. They also found changes in mucin expression in post-menopausal women and in women using LNG-IUD, but no differences in follicular versus luteal phase samples. Their results were notable in showing that the presence of bacterial vaginosis, a clinical condition reflecting a change in the vaginal microbiome, was strongly associated with alterations in glycosidases, glycans, and mucins in the cervicovaginal fluids. We controlled for this variable in our sample collection by checking vaginal pH and performing wet mount microscopy to exclude women with sub-clinical or clinical bacterial vaginosis.

In the cross-sectional validation cohort, we were able to compare samples from women with high endogenous progesterone (luteal phase for control and Cu-IUD groups) to those on the synthetic progestins levonorgestrel (COCs and LNG-IUD) and DMPA, a long-acting progestin administered by injection. Given that the Cu-IUD is a non-hormonal contraceptive, we did not expect, nor did we observe, a difference in

the glycosylation patterns compared to controls, confirming that the Cu-IUD does not have an independent effect on cervical mucus quality over and above the effect of luteal phase progesterone levels. Interestingly, the glycan-binding patterns of the LNG-containing contraceptives were generally similar to each other and to those from women in the luteal phase. Conversely, the DMPA samples showed different binding patterns. It is well known that progestins have differences in their mechanisms of action, for example, DMPA has a stronger glucocorticoid profile than LNG; differences in steroid mechanisms of action are presumably reflected in different carbohydrate fingerprints in cervical mucus. Further exploration of these differences can be performed with proteomic and glycomic analyses and will be useful in identifying mechanisms of action of different progestins on cervical mucus.

The lectins LCA and TL and the antibody MECA-79 all detected ligands on a 164-kD band present at early time points in the menstrual cycle. This result suggests that there is a protein that expresses all three glycan moieties; alternatively, different proteins, each with different carbohydrates, could co-migrate at the same molecular weight. Proteomic analysis of the high molecular weight bands will resolve these possibilities.

A strength of our study is the use of samples from defined stages of the menstrual cycle, to control for hormonal fluctuations that could influence results. The longitudinal cohort allowed direct comparison of binding patterns from the same woman at different points in the menstrual cycle, limiting effects of person-to-person confounders. A limitation of this study is the small number of samples; we aimed to have samples from ten women in the discovery cohort. However, the validation of most of our findings with the independently collected samples in the cross-sectional cohort from women in the luteal phase is reassuring that our results are robust and reproducible despite the small sample size and sample collection extending for 6 years. Another potential limitation of our study is that given the sample collection technique, we analyzed only soluble proteins; any significant glycosylation changes occurring on crosslinked insoluble mucins would not be detected by this approach.

These results indicate that cervical wick samples are a unique and previously under-utilized source of cervical mucus that is amenable to biochemical analyses to identify cycle-dependent changes in glycosylation. Application of comprehensive proteomic and glycomic techniques to well-annotated wick samples collected at the time of ovulation (high estrogen, favorable mucus) and in the luteal phase (high progesterone, unfavorable mucus) will allow identification of molecular signatures of the cervical factor in fertility and contraception. Such biomarkers will be useful for developing diagnostics to identify the fertile window for conception as well as identifying druggable targets for non-hormonal contraceptive discovery.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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