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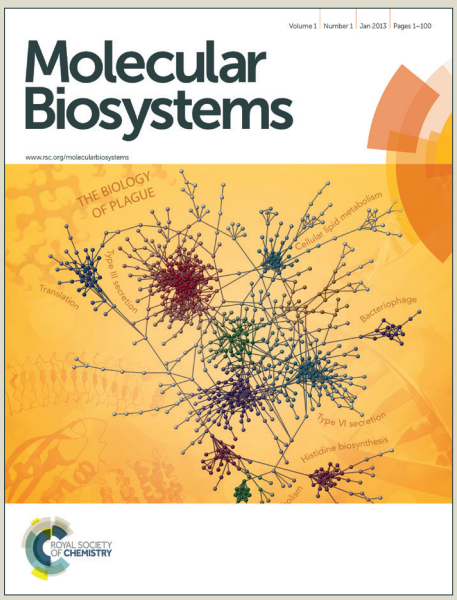

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ARTICLE

Proteomic characterization of the qualitative and quantitative differences in cervical mucus composition during the menstrual cycle

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The chemical composition of the cervical mucus (CM), its physical characteristics and the volume secreted change cyclically throughout the menstrual cycle. Aim of this study was to identify the constitutive protein compostition of CM of fertile women and the changes in the CM proteome throughout the menstrual cycle. Five fertile women who had a term delivery within 1 year before the study were enrolled. Proteomic analysis was performed by an Ultimate 3000 Nano/Micro-HPLC apparatus equipped with an FLM-3000-Flow manager module and coupled with an LTQ Orbitrap XL hybrid mass spectrometer; bioinformatic software were used for functional and quantitative analysis. 59, 81 and 43 proteins (mean) were respectively identified in the pre-ovulatory, ovulatory and post-ovulatory samples. 38 common proteins were identified. 42, 38 and 17 exclusive proteins were respectively identified in pre-ovulatory, ovulatory and post-ovulatory CM. The main part of CM constituents has a catalytic activity, which is mainly related to hydrolase activity. The label-free quantitative analysis for the common proteins revealed a significant reduction in the protein abundance index for antileukoproteinase, after the ovulation, and a peak of haptoglobin at ovulation. This is the first application of high-resolution MS-based proteomics for the identification of protein constituents of CM. This approach may contribute in identifying putative biomarkes of the female reproductive tract.

Introduction

The secretory cells of the glands in the cervical crypts produce cervical mucus (CM), that facilitates sperm penetration into the uterus during ovulation. The ovulatory CM provides in fact a defense for the sperm against the hostile acidic vaginal secretions, fulfills its energy demands, filters the ejaculate of the morphologically abnormal and dismotile sperm and provides conditions for sperm capacitation (1). Moreover the CM represents an inhibitory microenvironment for sperm propagation during the non-ovulatory phases of the cycle. However the function of cervical mucus is not yet fully understood.

The chemical composition of the CM is important for the fertility, since the addition of mucospissic agents and the presence of antisperm antibodies in human mid-cycle CM may transform CM from a sperm-receptive to a sperm-hostile element (2).

The chemical composition of the CM, its physical characteristics and the volume secreted change cyclically throughout the menstrual cycle. During the first half of the cycle the mucus is scant, thick and viscous, forming a physical barrier that limits the access of sperm to the upper genital tract. Then, immediately before ovulation, when estrogens are produced in increasing amounts, the cervical mucus viscosity decreases while the volume increases 10-20 folds, thereby maximizing its permeability to sperm. Immediately after ovulation, the quality of cervical mucus changes when the corpus luteum of the ovary begins to synthesize progesterone. Progesterone induces a reduction in the quantity and quality of CM, that becomes thicker and sticker (3).

The cyclic variability of cervical mucus has crucial, but not yet fully understood, impact on fertility and reproductive health. The characteristics of the CM are moreover, an important diagnostic marker for estimating the time of ovulation (4-6). It has been used by women from many nations and cultures to

recognize the fertile phase for the purpose of fertility awareness (7).

Due to the very complex chemical and biophysical structure of cervical mucus, the available data are frequently incomplete and sometimes conflicting.

Mucins are the most abundant proteins near ovulation while the abundance of other proteins increases during the luteal phase (8). Wolf et al. proposed that the variable viscoelastic properties of cervical mucus are determined by its carbohydrate composition and/or structure. They also suggested that mucin concentration is dependent on variations in the hormonal milieu (9). Previous studies have shown the characteristics of mucin aggregates by means of atomic force microscopy and demonstrated the switch from fibrous preovulatory to globular ovulatory mucus (10).

In the postgenomic era, proteomic technology has rapidly developed becoming a powerful tool in the research of human physiology, in particular in biological fluid, characterizing the comprehensive proteomic composition and identifying potential novel biomarkers for diagnosis, prognosis and therapy in different clinical aspects, including reproduction (11).

A comprehensive human cervical mucus proteome catalog is still missing, even though the material is generally accepted as a biofluid. The proteome composition of cervical mucus is probably less complex when compared to plasma and urine but how many proteins cervical mucus contains and how the cervical mucus proteome undergoes to cyclical changes remain unclear.

Few studies have in fact investigated the proteic composition of cervical mucus. The presence of mucins and of anti-microbial peptides were previously reported in the cervical mucus plugs during pregnancy (12). More recently, 137 common proteins were identified in a pool of five cervical mucus plugs obtained from women in labor at term, by using LC–MS/MS (13).

Andersch-Björkman et al. have reported proteomic and glycomic analyses of the cervical mucus obtained from 12 non-pregnant women using LC-FT-ICR and MS/MS (14). They performed a pre-fractionation of the samples by one-dimensional SDS-PAGE and SDS-Agarose composite gel electrophoresis for the separation of proteins and mucins with subsequent tryptic analysis; major result was related to an interesting alterations in the mucin glycans. Moreover Panicker et al. applied SELDI-TOF MS in order to study CM composition (15).

The aim of this study was to investigate proteomic changes of CM at different phases of the menstrual cycle by an LTQ-Orbitrap XL mass spectrometer, implemented by bioinformatic tools for functional and quantitative analysis.

Results and discussion

In this study modern and more specific methodologies of high resolution mass spectrometry and bioinformatics analysis were utilized in order to determine the in vivo changes of CM proteic composition during the menstrual cycle.

The biochemical analysis of the intact CM is difficult because of its extremely high viscosity. To reduce viscosity cervical mucus was diluted in aqueous TFA solution. The acidic environment generated by the aqueous TFA, in addition, decreased protein degradation induced by proteinases after collection. This treatment facilitated the solubilization of small peptides that could otherwise co-precipitate with high molecular weight proteins, such as mucins, that were excluded by this procedure from the analysis. This approach can be adopted as the standard protocol for future studies on CM proteome.

Protein identification using stringent criteria lead to the characterization of a mean of 59 proteins in the pre-ovulatory samples, 81 proteins in the ovulatory samples and 43 proteins in the post-ovulatory samples.

The increase in the number of proteins at ovulation suggests that the major synthesis of proteins in CM is induced at the moment of ovulation and might be the consequence of the increase in estrogen levels.

Thirty-eight proteins were identified in all the 3 phases of the menstrual cycle. The complete list of these proteins, considered as constitutive of the CM in all the phases is reported in Table 1. For each protein we reported the following informations: UniPROT, Gene name, molecular weight (MW), isoelectric point (pI) and Description.

Table 1: Constitutive CM proteins

UniPROT	Gene name	MW [kDa]	calc. pI	Description
P60709	ACTB	11.3	7.24	Actin
P04217	A1BG	69.3	6.28	Alpha-1B-glycoprotein
P03973	SLPI	15.2	8.68	Antileukoproteinase
P02647	APOA1	16.0	7.28	Apolipoprotein A-I
Q9UBG3	CRNN	11.6	5.87	Cornulin
P00738	HPT	16.0	8.05	Haptoglobin
P69905	HBA	13.2	6.13	Hemoglobin subunit alpha
P68871	HBB	11.2	7.24	Hemoglobin subunit beta
P02042	HBD	18.1	8.57	Hemoglobin subunit delta
P02790	HEMO	14.3	8.75	Hemopexin
P23527	H2B1O	16.5	9.16	Histone H2B type 1-O
P01876	IGHA1]	37.6	6.51	Ig alpha-1 chain C region
P01877	IGHA2	77.0	7.12	Ig alpha-2 chain C region
P01857	IGHG1	36.5	6.10	Ig gamma-1 chain C region
P01859	IGHG2	13.9	10.32	Ig gamma-2 chain C region
P01860	IGHG3	36.1	8.19	Ig gamma-3 chain C region
P01861	IGHG4	10.2	6.99	Ig gamma-4 chain C region
P01834	IGKC	13.0	4.84	Ig kappa chain C region
P01620	KV302	35.9	7.59	Ig kappa chain V-III region SIE
P01717	LV403	15.0	8.27	Ig lambda chain V-IV region Hil
P0CG05	LAC2	5.0	5.06	Ig lambda-2 chain C regions
P0CG06	LAC3	22.6	8.91	Ig lambda-3 chain C regions
P01591	IGJ	78.1	8.12	Immunoglobulin J chain
O60282	KIF5C	41.3	7.90	Kinesin heavy chain
P05164	PERM	45.2	6.58	Myeloperoxidase
P02788	TRFL	35.9	7.36	Lactotransferrin
P61626	LYSC	41.7	5.48	Lysozyme C
P59665	DEF1	18.1	5.24	Neutrophil defensin 1

P80188	NGAL	53.5	6.10	Neutrophil gelatinase-associated lipocalin
P07737	PROF1	11.8	8.48	Profilin-1
P06702	S10A9	73.8	9.11	Protein S100-A9
Q4W5P6	TM155	11.5	6.51	Protein TMEM155
P20848	A1ATR	30.8	5.76	Putative alpha-1-antitrypsin-related protein
P02787	TRFE	54.2	5.86	Serotransferrin
P02768	ALBU	14.2	10.26	Serum albumin
Q9UBC9	SPRR3	51.6	7.02	Small proline-rich protein 3
P62328	TYB4	47.9	7.90	Thymosin beta-4
Q14508	WFDC2	83.1	6.20	WAP four-disulfide core domain protein 2

The list of the identified peptides for each common and phase-specific protein is reported as supplementary matherial (S1).

The reported list of these 38 proteins could represent the constitutive CM proteomic profile, independent from the hormonal control. The significance of these proteins in both basic and applied female reproduction can now be explored and is certainly worthy of further investigation.

Among the 38 constitutive proteins, the presence of immunoglobulin chains, neutrophil defensin 1, WAP four-disulfide core (WFDC) domain protein 2 and lactotransferrin suggests the importance of the defence role of CM.

Due to the presence of immunoglobulin-producing cells in the uterine cervix, the mucus contains secreted antibodies (16,17), that binds pathogens and facilitate their trapping into the network of mucins. IgA and IgG are differentially and specifically associated with the different types of mucus of the female reproductive tract: both IgA and IgG are stably associated with cervical mucus, but only IgGs are associated with the cervico-vaginal mucus (18). The loss of IgA in the lower genital tract may be the consequence of changes in CM interactions with mucins or other proteins associated with mucus.

Human defensins are small cationic antimicrobial polypeptides and include the α-defensin human neutrophil peptides 1 to 3 (HNP1-3), that are released from the neutrophils (19).

WFDC motifs are composed of approximately 50 aminoacids including eight cysteine residues at defined positions, which form four disulfide bonds. This motif is present in a number of other secreted proteins, including elafin, eppin, and antileukoproteinase, which form the WFDC family of proteins. Initially, it was thought that the biological role of these proteins was to regulate inhibition of a wide spectrum of microbial and leucocyte proteolytic enzymes (20). There are at present several published data demonstrating that WFDC domains may also possess, in addition to the antiprotease activity, other properties such as antibacterial, anti-fungal, antiviral and anti-inflammatory functions (21).

Lactotransferrin, also called lactoferrin, is a mammalian iron scavenging defense protein, constitutively present in secretions that are consistently exposed to microbial flora: milk, tears, tubotympanum and nasal exudate, saliva, bronchial mucus, gastrointestinal fluids, cervical mucus and seminal fluid. The main function of lactoferrin is to scavange non-protein-bound iron in body fluids (22) and to suppress oxidative stress-mediated damage (23). The secretion of humoral soluble factors in cervical mucus such as lactoferrin, represents a first non immune defence involved in preventing ascending infections.

Further studies are needed in order to understand the interactions between immunoglobulins, defense and binding proteins and other CM proteins, which might open the perspective to the development of vaccines designed to protect the mucosal barriers from sexually transmitted diseases.

Comparative analysis for the identification of phase-specific proteins lead to the identification of 42 specific proteins in the pre-ovulatory phase, 38 specific proteins in the ovulatory phase and 17 specific proteins in the post-ovulatory phase (Table 2). For each protein we reported the following informations: UniPROT, Gene name, MW, pI and description.

Table 2: Phase-specific proteins

UniPROT	Gene name	MW [kDa]	calc. pI	Description
		PRE-OVULATORY SPECIFIC PROTEINS		
Q9Y2I7	FYV1	10.9	5.78	1-phosphatidylinositol 3-phosphate 5-kinase
P04083	ANXA1	15.8	8.75	Annexin A1
O95994	AGR2	9.9	5.06	Anterior gradient protein 2 homolog
P02652	APOA2	15.9	5.76	Apolipoprotein A-II
P02749	APOH	7.4	8.60	Beta-2-glycoprotein 1
P60022	DEFB1	11.2	6.62	Beta-defensin 1
Q8TDL5	BPIB1	8.6	7.25	BPI fold-containing family B member 1
Q9NPI1	BRD7	29.8	7.97	Bromodomain-containing protein 7
Q9NRJ3	CCL28	23.2	8.10	C-C motif chemokine 28
P08123	CO1A2	17.0	7.24	Collagen alpha-2(I) chain
P02748	CO9	20.0	9.00	Complement component C9
P08603	CFAH	21.0	7.53	Complement factor H
P01034	CYTC	14.6	10.04	Cystatin-C
Q8NCM8	DYHC2	38.2	6.95	Cytoplasmic dynein 2 heavy chain 1
P68104	EF1A1	19.0	7.84	Elongation factor 1-alpha 1
P15328	FOLR1	52.4	7.23	Folate receptor alpha
Q08380	LG3BP	18.7	9.09	Galectin-3-binding protein
P80748	LV302	65.3	5.27	Ig lambda chain V-III region LOI
P22692	IBP4	27.9	7.15	Insulin-like growth factor-binding protein 4
Q8N6S4	AN13C	37.1	6.84	Ankyrin repeat domain-containing protein 13C
Q9Y4D1	DAAM1	31.2	6.43	Disheveled-associated activator of morphogenesis 1
Q16270	IBP7	32.3	6.65	Insulin-like growth factor-binding protein 7
Q03405	UPAR	14.3	10.20	Urokinase plasminogen activator surface receptor
P10909	CLUS	50.1	9.01	Clusterin
P09466	PAEP	38.3	7.97	Glycodelin

Q9XRX5	HHLA3	11.9	5.08	HERV-H LTR-associating protein 3
Q99996	AKAP9	38.7	7.02	A-kinase anchor protein 9
Q8N4C8	MINK1	28.8	8.12	Misshapen-like kinase 1
Q9Y6F6	MRVI1	63.1	5.59	Protein MRVI1
P30044	PRDX5	65.8	5.43	Peroxiredoxin-5
P02750	A2GL	57.0	6.87	Leucine-rich alpha-2-glycoprotein
Q38SD2	LRRK1	129.2	8.95	Leucine-rich repeat serine/threonine-protein kinase 1
O75556	SG2A1	91.5	6.34	Mammaglobin-B
P01033	TIMP1	191.2	4.89	Metalloproteinase inhibitor 1
Q6UB28	MAP12	74.1	6.39	Methionine aminopeptidase 1D
Q96PE7	MCEE	122.2	7.23	Methylmalonyl-CoA epimerase
Q12851	M4K2	174.6	6.89	Mitogen-activated protein kinase kinase kinase kinase 2
Q13232	NDK3	139.0	6.61	Nucleoside diphosphate kinase 3
P30086	PEBP1	143.5	7.12	Phosphatidylethanolamine-. binding protein 1
P37108	SRP14	492.3	6.54	Signal recognition particle 14 kDa protein
P02766	TTHY	225.2	6.68	Transthyretin
Q9NYU1	UGGG2	237.0	6.70	UDP-glucose:glycoprotein glucosyltransferase 2

OVULATORY SPECIFIC PROTEINS

P01023	A2MG	67.06	6.04	Alpha-2-macroglobulin
P18054	LOX12	75.65	6.21	Arachidonate 12-lipoxygenase, 12S-type
Q5TGY3	AHDC1	54.81	6.61	AT-hook DNA-binding motif-containing protein 1
Q5PSV4	BRM1L	67.75	4.79	Breast cancer metastasis-suppressor 1-like protein
Q08493	PDE4C	347.38	6.20	cAMP-specific 3',5'-cyclic phosphodiesterase 4C
Q8IYX3	CC116	115.76	5.49	Coiled-coil domain-containing protein 116
Q8N4S0	CCD82	37.61	5.15	Coiled-coil domain-containing protein 82
P12109	CO6A1	92.25	6.86	Collagen alpha-1(VI) chain
Q8WTW3	COG1	63.96	5.01	Conserved oligomeric Golgi complex subunit 1
P35462	DRD3	275.48	7.24	D(3) dopamine receptor
P32926	DSG3	40.73	8.54	Desmoglein-3
Q8TEC5	SH3R2	180.56	5.99	E3 ubiquitin-protein ligase SH3RF2
Q9P2K8	E2AK4	104.59	6.07	Eukaryotic translation initiation factor 2-alpha kinase 4
Q5XX13	FBW10	379.64	8.51	F-box/WD repeat-containing protein 10
P02751	FINC	238.73	6.68	Fibronectin
Q5CZC0	FSIP2	174.87	5.60	Fibrous sheath-interacting protein 2
P08107	HSP71	222.84	5.68	Heat shock 70 kDa protein 1A/1B
P42858	HD	107.47	5.00	Huntingtin
Q9BYI3	HYCCI	66.52	9.04	Hyccin
Q8NI35	INADL	131.63	6.71	InaD-like protein
P27816	MAP4	168.24	9.04	Microtubule-associated protein 4
Q13164	MK07	56.29	8.37	Mitogen-activated protein kinase 7
O43148	MCES	108.46	5.43	mRNA cap guanine-N7 methyltransferase
Q13765	NACA	56.90	9.64	Nascent polypeptide-associated complex subunit alpha
O94964	SOGA1	226.55	6.20	Protein SOGA1
O14795	UN13B	94.61	5.10	Protein unc-13 homolog B
Q9Y3P9	RBGP1	97.88	6.38	Rab GTPase-activating protein 1
P08240	SRPR	106.44	6.48	Signal recognition particle receptor subunit alpha
Q96PX8	SLIK1	332.97	7.28	SLIT and NTRK-like protein 1
Q9H3E2	SNX25	243.79	6.89	Sorting nexin-25
Q86TI0	TBCD1	163.19	6.46	TBC1 domain family member 1
P23458	JAK1	113.90	9.38	Tyrosine-protein kinase JAK1
Q9NYU2	UGGG1	73.17	5.69	UDP-glucose:glycoprotein glucosyltransferase 1
Q92614	MY18A	133.00	6.96	Unconventional myosin-XVIIIa
Q6ZQQ6	WDR87	162.93	7.68	WD repeat-containing protein 87
Q86UP3	ZFHX4	125.15	4.88	Zinc finger homeobox protein 4
Q9Y6X8	ZHX2	379.87	8.34	Zinc fingers and homeoboxes protein 2
Q9H4I2	ZHX3	253.27	6.60	Zinc fingers and homeoboxes protein 3

POST-OVULATORY SPECIFIC PROTEINS

Q6SPF0	SAMD1	56.0	7.58	Atherin
Q8WXX0	DYH7	57.6	7.36	Dynein heavy chain 7
Q9NS56	TOPRS	112.3	9.41	E3 ubiquitin-protein ligase Topors
Q14576	ELAV3	39.5	9.28	ELAV-like protein 3
Q5VW36	FOCAD	199.9	6.62	Focadhesin
P48637	GSHB	40.3	5.11	Glutathione synthetase
Q6UXS9	CASPC	29.4	6.21	Inactive caspase-12
P01579	IFNG	19.3	9.47	Interferon gamma
P13645	K1C10	58.8	5.21	Keratin, type I cytoskeletal 10
P05787	K2C8	53.7	5.59	Keratin, type II cytoskeletal 8
Q99583	MNT	62.3	8.78	Max-binding protein MNT
P18669	PGAM1	28.8	7.18	Phosphoglycerate mutase 1
P00734	THRB	70.0	5.90	Prothrombin
Q9C0H5	RHG39	121.2	7.50	Rho GTPase-activating protein 39
Q13813	SPTN1	282.1	5.34	Spectrin alpha chain, non-erythrocytic 1
P60174	TPIS	17.9	5.58	Triosephosphate isomerase
Q86VK4	ZN410	44.2	6.57	Zinc finger protein 410

These data seem apparently in contrast with the previously data reported by Andersch-Bjorkman et al (15). They reported in fact in 2007 that, when other proteins than mucins were separated by gel electrophoresis, the silver-stained protein profiles from different cycle days revealed a common pattern and no significant differences have been reported. Their major result were related to an interesting alterations in the mucin glycans. On the contrary, we preferred a different procedure excluding mucins from analytical plan by TFA treatment and performing one single shotgun analysis for each sample, using a mass spectrometer of similar sensitivity. Our experimental approach led us to obtain evidence on alterations of protein pattern over the different menstrual phases. We hypothesize

that the main reason of this discrepancy may rely on the different sample treatment.

In the pre-ovulatory CM, 42 specific proteins were identified including cystatin C, clusterin, glycodelin, metalloproteinase inhibitor 1.

Cystatins are cysteine protease inhibitors, found in a wide range of organisms (24). Cystatin-C was identified for the first time in CM as a marker of the pre-ovulatory phase.

Clusterin is a protein previously identified in different secretions, including milk (25) and seminal plasma (26). It is related to the damaging oxidative reactions (27) and protein precipitation (28). Its role in reproduction is mainly related to the control of the agglutination of abnormal spermatozoa (29).

Glycodelin was reported to be involved in inhibiting sperm capacitation and function (30). The correlation between CM proteins, sperm function and reproduction represents an interesting field for future studies.

There are some literature reports about the inhibitors of metalloproteinases (TIMP) in reproduction, linking the expression of metalloproteinases-TIMPs system in the cervical mucus plug during pregnancy with the proteolytic activity in connection with term and pre-term birth (31). The significance of the exclusive presence in the pre-ovulatory CM of TIMP-1 might be associated to the inhibition of proteolitic activity, which leads to the liquefaction of CM in the ovulatory phase.

In the post-ovulatory phase 17 specific proteins were identified, including glycolytic enzymes such as triosephosphate isomerase (TPI) and structural proteins such as keratin I and II, spectrin and dynein chains which are essential for the manteinance and organization of intermediate filament network.

Sperm movement modifications are depending on external regulatory factors, some of which are secreted by the cervical glands of the cervix, by the oocyte or by the perioocyte layers to create a sperm chemotaxis which would optimize the gametic interaction (32). Previous data demonstrated that TPI is also localized at the acrosomal level in human spermatozoa (33) and that TPI is involved in the sperm-oocyte interaction. TPI is an enzyme that catalyzes the interconversion of dihydroxyacetone phosphatase and glyceraldehydes-3-phosphatase, completing the preparatory phases of glicolysis. TPI moreover contributes to cytoskeleton regulation interacting with Rho in the regulation of intracellular sodium through Na,K–ATPase activation, by providing glycolitic ATP that fuels energy for membrane function (34). TPI is associated with the plasma membrane and binds indirectly to structural proteins such as actin ad microtubules, which are involved in the microfibrillar structure of the CM, which constraints the flagellar wave amplitude of sperm.

Dynein chain might be associated with the exocytosis of the cervical granules, which could contribute in the changes in biochemical and microfibrillar pattern of CM.

Finally, we reported the presence of E3 ubiquitin-protein ligase SH3RF2 in the ovulatory CM and of the E3 ubiquitin-protein ligase Topors in the post-ovulatory phase. Ubiquitin is a 76-residue protein, involved in protein degradation by the ubiquitin-proteasome system. Ubiquitilation is important in processes such as apoptosis and antigen presentation, and represents a central process in oncogenesis, including the development of cervical neoplasia (35). Here we reported the first identification of enzymes involved in ubiquitinilation in CM that can be associated to the selection of the spermatozoa.

The bioinformatic analysis for the molecular functions of the common proteins, as reported in figure 1, revealed that the main part of CM constituents have a catalytic activity (36%), which is mainly related to hydrolase activity (60% of the catalytic group). CM possesses in fact a considerable quantity of enzymes involved in mucin breakdown as proteases, sialidases, other glycosidases and sulphatases. Their activities change mucin structure and hence its physical properties. These enzymes may influence the physical barrier to sperm cells and pathogens or may enhance bacterial adhesion and hence colonization (36) and might be moreover involved in enzymatic processes associated with sperm capacitation.

Binding proteins represent the second group for molecular activity classification (31%), mainly involved in protein-protein interactions (50% of the binding proteins).

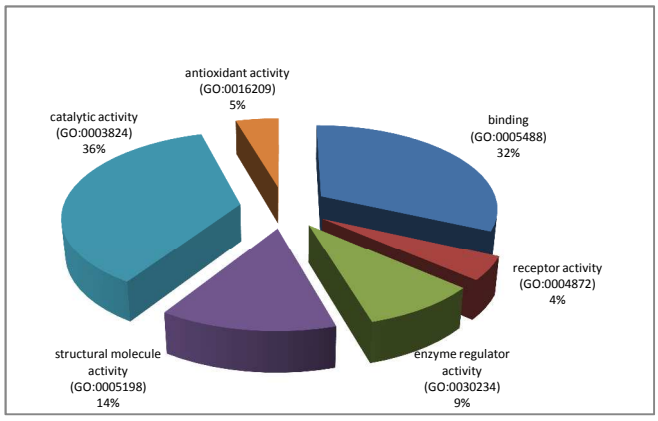

Figure 1: *Analysis of common proteins according to GO Molecular Function annotations*

The comparative analysis of GO annotations for molecular function for the exclusive proteins identified in pre-ovulatory and ovulatory CM reported significative differences in some important protein classes such as an increase of defense/immunity proteins at the ovulatory phase. This data suggests an associations between estrogens and the protective capacity of cervical mucus. This is consistent with previous studies demonstrating that the expression of factors reducing Candida Albicans binding to vaginal epithelial cells are most prominent in the glandular epithelium of the endocervix during estrus and is stimulated by estradiol treatment in mice (37).

The comparison analysis of GO annotations for molecular function for the exclusive proteins identified in ovulatory and post-ovulatory CM reported a significative increase after the ovulation in some protein classes such as: G-protein coupled receptors, calcium binding proteins, hydrolases, protein kinases and methyltransferase.

This is the first study in which bioinformatic functional analysis of proteomic data offers additional information regarding the changes in CM functions during the menstrual cycle.

Finally, we applied for the first time a label free quantitative proteomic approach evaluating the differences in term of protein abundance for each identified protein between the different phases. This approach take in account the area of the three most abundant peptides, based on the premise that the MS signals of the most efficiently ionized peptides directly correlate with the corresponding protein amount. This method was originally proposed by Silva et al. (38) for Q-ToF instruments, but has been validated recently also for ion-trap based mass spectrometers running in data-dependent acquisition (DDA) mode (39).

The quantitative analysis for the common proteins revealed a reduction in the protein abundance index for antileukoproteinase, after the ovulation, and a peak of haptoglobin at the ovulation, which is reduced in pre-ovulatory and post-ovulatory CM.

This is consistent with previous literature data, demonstrating that ALP concentration is significantly higher in the ovulatory phase than in the follicular and luteal ones (40). ALP, also called secretory leukocyte protease inhibitor (SLPI), is a mucosal secreted protein with a low molecular-weight, which inhibits the activities of the neutral serine lysosomal proteinases, elastase and cathepsin-G, contributing to local protection against epithelial cell destruction (41). Potential regulators of pig endometrial SLPI gene expression include the steroid hormones, estrogen (E) and progesterone (P4) (42). Interestingly, E and P4 regulation of SLPI synthesis appears to be specific to the endometrium. In humans, SLPI was demonstrated to be present in the CM during all the menstrual cycle, with a peak at ovulation, as confirmed by our quantitative proteomic analysis.

A peak of haptoglobin (HP) was verified at ovulation. HP is an acute phase alpha-sialoglycoprotein with hemoglobin-binding capacity. Hemoglobin-HP complexes are removed as a consequence of the binding with the CD163 receptor, which is expressed on the surface of monocytes and macrophages. HP also have been demonstrated to modulate several aspects of the innate and adaptive immune response and to have anti-inflammatory activities (43). The quantitative increase of haptoglobin at ovulation as reported by quantitative proteomic analysis is consistent with the observed increase in defence activity, which we observed by functional protein annotation.

Although our present results may provide a basis for a better understanding of the role of CM in reproduction, we must also acknowledge certain limitation of our experimental design. First, we performed our study on a relatively small sample scale. Second, the study was based solely on proteomic data, not confirmed by other techniques.

However this approach has permitted, by high resolution mass spectrometry, the detection of an array of proteins in cervical mucus reflecting both the constitutive composition and the changes during the menstrual cycle.

Experimental

The design of the study was approved by the Institutional Review Board of our University.
Five 25-30 year-old fertile women, with no history of fertility problems and who had a term delivery within 1 year before the study were enrolled. The subjects gave informed consent according to the guidelines of the Declaration of Helsinki.
The subjects maintained sexual abstinence during the study. Vaginal and cervical swabs were obtained before sample collection to exclude vaginal and cervical infection. PAP test and colposcopy were also performed before sample collection.
Cervical mucus samples (n=3 per patient) were obtained by gentle aspiration from the cervical canal with a catheter for intrauterine insemination (Gynetics Medical Products, Achel, Belgium). Samples were collected before (day 7), during (day 12), and after (day 18) ovulation of the same menstrual cycle. Ovulation was assessed by transvaginal sonography and confirmed by measurement of midluteal serum progesterone levels.
The cervical mucus was collected in plastic tubes and mixed 1:1 (v/v) with aqueous trifluoroacetic acid solution (0.2% v/v) and centrifuged at 9200xg for 10 minutes. The soluble acidic fraction was stored at -80°C until analysis.
An aliquot of the soluble acidic fraction of each CM sample, corresponding to 0.5 mg of total protein (as measured by Bradford assay), was mixed with 1 M ammonium bicarbonate pH 8.0 and reduced with 200 mM dithiothreitol (DTT 10 mM final, Sigma) for 5 minutes at 100°C and 15 minutes at 50°C, and then alkylated with 200 mM iodoacetamide (IAA 55mM final, Sigma) in the dark at room temperature for 60 minutes. The samples were left to digest overnight at 37°C by adding 100 mM ammonium bicarbonate (pH 8) with sequencing grade modified porcine trypsin (1:50, trypsin: protein concentration, Pierce Biotechnology). To stop the digestion, the samples were acidified with aqueous trifluoroacetic acid solution (0.2% v/v) and immediately frozen and lyophilized.
The samples were resuspended in 40 µl of aqueous formic acid solution (0.1% v/v) and analyzed by an Ultimate 3000 Nano/Micro-HPLC apparatus (Dionex, Sunnyvale, CA, USA) equipped with an FLM-3000-Flow manager module, and coupled with an LTQ Orbitrap XL hybrid mass spectrometer (ThermoFisher, San Jose, CA). Separations were performed by a Zorbax 300SB-C18 column (3,5 µm particle diameter; column dimension 1mm i.d. x 15 cm) (Agilent Technologies, Santa Clara, CA) using the following eluents: (A) 0.05% (v/v) aqueous formic acid and (B) acetonitrile:water 80:20 with 0.05% (v/v) aqueous formic acid. The applied gradient was linear from 0 to 55% of solvent B in 40 min, at a flow rate of 80 µL/min. The LTQ-Orbitrap mass spectrometer was operated in a data-dependent mode in which each full MS scan (60000 resolving power) was followed by three MS/MS scans where the three most intense multiple-charged ions were dynamically selected and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35%.
Samples were analyzed individually; proteomic analysis was performed at the same time for all samples, while data analysis was subsequently performed.
Tandem mass spectra were analysed by the Thermo Proteome Discoverer 1.4 software, using SEQUEST cluster (University of Washington, Seattle, WA, licensed by Thermo Electron Corp) as search engine against UniProtKb/Swiss-Prot protein knowledgebase release 2013-02: 20247 Homo Sapiens protein database.

In order to obtain a reliable identification of the peptides, the following stringent filters were used: XCorr versus charge, 1.8 and 2.5 for doubly and triply charged ions respectively; high value peptide confidence, false discovery rate (FDR) <5% . Data were searched with three missed cleavages, fixed carbamidomethylation of cysteines and the oxidation of methionines as variable modification.
In order to identify a proteic pattern for cervical mucus we considered for this study the panel of proteins identified in at least one sample for each menstrual cycle phase (preovulatory, periovulatory and postovulatory) with an X score assigned by the software >1.5. Subsequently, in order to identify proteic markers for each cycle phase, we further considered the proteins exclusively identified in each ovulatory phase.
Proteins were reported as "phase-specific" when they have been identified in the samples of one specific menstrual phase but they were not present or under the instrumental limit of sensitivity in the other phases, so that they are anyway more abundant and characterizing each of the menstrual phase.
Proteins identified by SEQUEST were analyzed using the publicly available PANTHER (protein annotation through evolutionary relationship) software (http://www.pantherdb.org/). For the aim of this study, we evaluated the GO annotations for molecular function in the common proteins.
The differences between pre-ovulatory and ovulatory exclusive proteins and between ovulatory and post-ovulatory exclusive proteins were also evaluated according to GO classes for molecular function. Statistical significance was given for $p < 0.05$.
The label-free quantification of common proteins was performed using the Peak Area Calculation Quantification during the bioinformatic analysis by Proteome Discoverer software. This quantification method is used to obtain an idea of the relative quantities of all peptides in a sample. The Proteome Discoverer application calculates peptide areas during processing, using them to automatically calculate protein areas for the proteins in the report. It calculates the area of any given protein as the average of the three most abundant distinct peptides identified for the protein.
Statistical analysis was performed by the software MiniTab 1.7 (GMSL, Nerbiano (MI), Italia).

Conclusions

In summary our present proteomic approach reveals differences during the menstrual cycle in the expression of several proteins of the CM, that are involved in defense system, inflammation and structural activity. This work constitutes one of the few proteomic studies reported on CM asnd the first analysis of CM changes during the different phases of the menstrual cycle by high-resolution MS, implemented by quantitative tools.

Further characterization of proteins differentially expressed in CM may contribute to a better understanding of their role in reproduction, their effect on fertility and their biological regulation.

Notes

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F. Vincenzoni substantially contributed to the design of the work, through sample proteomic analysis and drafting the work.
G. Pompa substantially contributed to the design of the work, through clinical evaluation and cervical mucus collection. She moreover revised critically the manuscript for important intellectual content.
A. Biscione substantially contributed to the design of the work, through cervical mucus collection and sample preparation. She moreover revised critically the manuscript for important intellectual content.
A.L. Astorri substantially contributed to the design of the work, through clinical evaluation and cervical mucus collection. She moreover revised critically the manuscript for important intellectual content.
E. Fruscella substantially contributed to the design of the work, through clinical and ultrasound evaluation of the patients. She moreover revised critically the manuscript for important intellectual content.
A. De Luca substantially contributed to the design of the work, through sample preparation. She moreover revised critically the manuscript for important intellectual content.
I. Messana substantially contributed to the conception of the work. She moreover revised critically the manuscript for important intellectual content.
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