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Proteomic analysis of nipple aspirate fluid throughout the menstrual cycle in healthy pre-menopausal women

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Abstract A proteomic approach to nipple aspiration fluid (NAF) has been used in a number of studies comparing women with breast cancer and healthy women. However, to make useful comparisons between women with breast cancer and healthy women it is necessary to establish whether there is physiological variation in the proteomic profiles of NAF. The purpose of this study was, for the first time, to examine how the proteomic profile of NAF using surface-enhanced laser desorption ionisation time-of-flight mass spectrometry varies across the menstrual cycle in healthy pre-menopausal women. Twelve women were recruited and nipple aspiration was carried out weekly from both breasts of each subject for two menstrual cycles. Matching serum samples for luteinising hormone, follicle stimulating hormone and oestradiol were obtained at each aspiration attempt. Statistically significant peaks were found for three healthy volunteers ($p < 0.05$). However, the peaks that varied across the menstrual cycle were different from one healthy volunteer to another and the differences were small compared with the large variation in proteomic profiles between healthy volunteers. This study provides proof of concept that the NAF proteomic profile does not vary substantially during the menstrual cycle and that therefore it is valid to compare NAF profiles from pre-menopausal women that have been taken at different stages in the menstrual cycle.

Keywords Menstrual cycle · Nipple aspiration · Proteomics · SELDI-TOF/MS

Introduction

The advent of new molecular technologies such as proteomics has given rise to a number of studies using a proteomic approach to analyse nipple aspirate fluid (NAF) [1–4]. The majority of studies to date have compared the differences between women with breast cancer and healthy women [5–8] but little research has been carried out examining the normal physiology of NAF from healthy women.

An early study which extensively investigated the constituents of normal NAF [9] demonstrated that all normal classes of serum proteins were found in NAF but the proportions differed substantially from serum. A further study has examined the relationship between cytology [10], prostate-specific antigen (PSA) and the menstrual cycle [11] but as yet there are no studies examining changes in the proteomic profile with the menstrual cycle. As the body of work using a proteomic approach increases it becomes more important to establish the normal proteome of body fluids in order for the comparison between normal and a disease state to be made. In addition, women in whom the intraductal

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approach may be useful are young and pre-menopausal and therefore information regarding variation with the menstrual cycle is particularly relevant.

The purpose of this study was to examine how the proteomic profile of NAF using surface-enhanced laser desorption ionisation time-of-flight mass spectrometry (SELDI-TOF/MS) varies across the menstrual cycle in healthy pre-monopausal women. Consistent physiological changes, if present, could then be adjusted for during future studies comparing healthy women and women with breast cancer.

Materials and methods

Volunteers

Between June and September 2005, 12 healthy pre-menopausal volunteers were recruited to this study which was approved by the Royal Marsden Hospital Committee for Clinical Research and the Local Research Ethics Committee. Written informed consent was obtained. Exclusion criteria included a previous history of breast disease (excluding fibroadenoma and fibrocystic breast disease), current pregnancy, lactation within 12 months, women who are post-menopausal, previous peri-areolar surgery and current oral contraceptive use. Nipple aspiration was carried out weekly from both breasts of each subject for two menstrual cycles. Matching serum samples for luteinising hormone (LH), follicle stimulating hormone (FSH) and oestradiol (E_2) were also obtained at each aspiration attempt to determine the phase of the menstrual cycle.

Sample collection

Prior to nipple aspiration, the healthy volunteer carried out breast massage by applying moisturising lotion to the breast and massaging from the chest wall towards the nipple for 2 min. Following massage, the nipple was cleansed with an alcohol pad and then a cotton swab in order to remove keratin plugs. Nipple aspiration was then performed using a handheld suction device (Cytyc UK) attached to a 10-ml syringe. The cup was placed over the nipple and the plunger was withdrawn up to a maximum of 10 ml until NAF was visualised on the surface of the nipple. NAF was collected into 1.5 ml Eppendorf tubes (Sarstedt, Nümbrecht, Germany) and snap frozen on dry ice. Samples were stored at –80°C for up to 6 months. The samples underwent two freeze/thaw cycles for the CM10 array and three freeze/thaw cycles for the IMAC30 array. For very small quantities of NAF

(<3 μl) there was insufficient material to assay protein concentration and run the sample on two arrays and therefore these samples were discarded. If bilateral samples were taken, both were analysed.

Protein array experimental protocol

Weak cation exchange arrays (CM10) and metal chelator arrays (IMAC30) were obtained from Ciphergen Biosystems Inc. (Fremont, CA, USA). The IMAC30 ProteinArray is an immobilised metal affinity capture array which incorporates nitrilotriacetic acid groups and is capable of forming stable octahedral complexes with polyvalent metal ions including Cu^{2+}. The CM10 ProteinArray is a weak cation exchange array with a hydrophobic barrier coating. Prior to incubation on the arrays, the total protein concentration of each NAF sample was measured using the Quick Start Bradford protein assay (BioRad). Samples were diluted to 1 mg/ml with CM10 (50 mM ammonium acetate pH 4.0, 0.01% Triton X-100) or IMAC30 (0.1 M sodium phosphate, 0.5 M NaCl pH 7.0) binding buffer. For the IMAC30 array, 5 μl 0.1 M copper sulphate solution was applied to each spot and incubated for 10 min. The metal solution was removed and each spot was rinsed with 5 μl H_2O. A 0.1 M sodium acetate pH 4.5 (neutralisation buffer) was applied to each spot and incubated for 5 min. The neutralisation buffer was removed and each spot was rinsed with 5 μl H_2O. All arrays were then incubated with their respective binding buffers for three 5 min incubations. The binding buffer was removed and replaced with 5 μl of sample. The array was then incubated in a humid chamber for 1 h on a platform shaker at room temperature at 80 rpm. The sample was removed and each spot was washed three times with their respective binding buffers and incubated for 5 min each. Each spot was rinsed with 5 μl H_2O. The spots were allowed to air-dry for 10–15 min. One microlitre of sinapinic acid dissolved in 200 μl of 0.5% trifluoroacetic acid and 50% acetonitrile was applied to each spot and allowed to air-dry. This step was repeated a second time. Each sample was applied to the array in duplicate.

Molecules retained on the arrays were visualised by reading each array in a Ciphergen PBSII mass spectrometer (Ciphergen, Fremont, CA, USA). Each array was read using spot and array protocols generated in the ProteinArray Software program (Ciphergen). Arrays were read using a laser intensity of 220, detector sensitivity 8 and a focus mass 10,000 Da for the CM10 array and 15,000 Da for the IMAC30 array. Each spot was analysed from positions 20–80, with five position increments, and six shots per position, preceded by two

warming shots at 225. Mass accuracy was calibrated daily using the All-in-one peptide molecular mass standard (Ciphergen) which ensures accuracy to within 0.1% of protein mass. The coefficient of variation was calculated as 14% which is well below the value of 20% for accepted reproducibility in SELDI.

Data analysis

Spectra produced from each weekly sample from each healthy volunteer were collated and normalised. Following normalization, consistent peak clusters were generated using the Biomarker Wizard of ProteinArray Software (Ciphergen). The first pass used a signal-to-noise ratio set to five with a threshold of 20%. The second pass used a signal-to-noise ratio of two and included the same peaks and peaks within 0.3% of the mass of the peaks found in the first pass. Only the data below 3,000 Da were subjected to the Biomarker Wizard to avoid the matrix noise below 3,000 Da. The clusters of peaks generated were then analysed using the Kruskal–Wallis ANOVA test. Statistically significant peaks included those with a p-value <0.05.

Results

Production of NAF

Twelve female pre-menopausal volunteers (M01–M12) participated in the study over two menstrual cycles. The attendance and production of NAF are shown in Table 1. Of the 12 women, five (M01, M02, M06, M10 and M11) did not produce NAF on their first attendance and were therefore not subsequently recalled. Two women (M05 and M09) withdrew from the study after 1 and 3 weeks, respectively. The five remaining

women went on to complete 8 weeks of collection. Only one woman out of five produced NAF every week (M04), the other four women either did not produce NAF on one or more weeks or did not attend their appointments. Production of NAF was unpredictable for most of the women (Table 2). Healthy volunteer M04 was the most consistent and produced NAF from both breasts every week. Production from healthy volunteer M03 was unilateral on three occasions and no NAF was produced from either breast on one occasion. Healthy volunteer M06 produced no NAF or very small amounts of NAF for the first 3 weeks but production improved with time. Healthy volunteer M08 always produced NAF on the left but rarely on the right. Healthy volunteer M12 produced NAF from both breasts each week but did not attend for 2 weeks out of 8. The samples that were of sufficient volume to run on the arrays (≥3 μl) are shown in Table 3 together with the days of the menstrual cycle which were calculated from the last menstrual period of each healthy volunteer.

Data analysis

Four NAF samples were chosen to represent one menstrual cycle for each of the five healthy volunteers. For healthy volunteer M06, no statistically significant peaks were found on the IMAC30 array or the CM10 array. For healthy volunteer M08, there were no statistically significant peaks on the CM10 array and only one statistically significant peak at 4,295 Da on the IMAC30 array (data not shown). For healthy volunteer M03, no statistically significant peaks were found on the CM10 array and two statistically significant peaks were found on the IMAC30 array. Of the IMAC30 peaks, the peak at 4,161 Da can be seen to change in amplitude over the 4 weeks shown (Fig. 1a). If day -4 and day

Table 1 Attendance and production of NAF from the 12 volunteers, M01–M12

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
M01	N								
M02	N								
M03	Y	Y	Y	dna	Y	N	Y	Y	Y
M04	Y	Y	Y	Y	Y	Y	Y	Y	
M05	Y	W							
M06	N	Y	dna	N	Y	Y	Y	N	
M07	N								
M08	Y	dna	Y	Y	Y	Y	Y	Y	Y
M09	Y	Y	Y	W					
M10	N								
M11	N								
M12	Y	dna	Y	Y	Y	dna	Y	Y	

Y stands for ≥3 μl NAF produced by either breast, N stands for both breast produced <3 μl NAF, W stands for withdrew from study, *dna* stands for did not attend

Table 2 Volume (μl) of NAF collected from each breast of volunteers M03, M04, M06, M08 and M12

	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8		Week 9	
	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
M03	10	0	5	5	5	5	X	X	5	10	3	0	0	5	2	10	1	5
M04	50	100	12	150	15	190	50	100	10	150	10	150	10	100	5	100		
M06	0	2	5	0	X	X	2	1	5	5	3	3	5	5	1	1		
M08	5	0	X	X	5	3	7	1	5	0	5	3	5	0	3	0	5	1
M12	5	20	X	X	5	15	5	10	5	10	X	X	5	10	5	10		

L left breast, *R* right breast, *X* did not attend

Table 3 Nipple aspiration fluid samples that were analysed by SELDI-TOF/MS and serum hormone levels for each volunteer

M03							M08						
Day	MC	L	R	LH	FSH	E$_2$	Day	MC	L	R	LH	FSH	E$_2$
1		X		5	4	527	1		X				
8		X	X	3	20	142	7						
13		X	X	4	12	545	15		X		2	2	225
20							22	3	X		2	9	56
28	-4	X	X	4	5	273	30	11	X		3	5	356
36							36	17	X	X	8	11	241
42	11		X	5	6	1,043	43	24	X		<1	3	208
49	21		X	2	3	536	50		X		3	11	125
56	27		X	1	3	99	57		X		5	5	330

M04							M12						
Day	MC	L	R	LH	FSH	E$_2$	Day	MC	L	R	LH	FSH	E$_2$
1		X	X	5	3	812	1		X	X	6	5	515
10		X	X	5	2	452	7						
15	-3	X	X	4	3	526	13	-5	X	X	4	6	160
26	9	X	X	6	5	199	20	3	X	X	4	4	266
32	15	X	X	33	9	559	27	10	X	X	7	7	476
38	21	X	X	<1	2	325	34	23					
45		X	X				40		X	X	4	6	309
51		X	X	3	5	153	47			X	6	5	

M06						
Day	MC	L	R	LH	FSH	E$_2$
1						
8		X				
16				18	12	294
22				3	4	696
29	-1	X	X	4	5	302
37	8	X		5	7	264
43	14	X		67	20	1,027
50	21			4	4	499

First column shows day of sample collection, second column shows day of menstrual cycle, X indicates samples that were analysed by SELDI-TOF/MS

L left breast, *R* right breast, *LH* luteinizing hormone (U/l), *FSH* follicle stimulating hormone (U/l), *E$_2$* oestradiol (pmol/l)

21 are compared, this peak falls to approximately half its' amplitude but an adjacent peak remains constant. This confirms that only certain peaks change in amplitude and rules out an error such as a lower protein concentration of NAF on day 21. Adjacent peaks can also be seen to vary in amplitude but these peaks are not statistically significant. For healthy volunteer M12, there was one statistically significant peak at 11,259 Da on the IMAC30 array (Fig. 1b) and no statistically significant peaks on the CM10 array. Finally, for healthy volunteer M04, two statistically significant peaks were found on the CM10 array (data not shown) and eight statistically significant peaks were found on the IMAC30 array. Of these eight IMAC30 peaks, five are shown in Fig. 2 to illustrate the change in amplitude over the 4 week period. If day 15 is compared to the other 3 days, the peak at 5,268 Da is seen at day 15 but is not present on days -3, 9 or 21.

Fig. 1 Statistically significant peaks in healthy volunteers M03 and M12 on the IMAC30 array. Spectra showing a statistically significant peak from **a** healthy volunteer M03 on days -4, 11, 21 and 27 and **b** from healthy volunteer M12 on days -5, 3, 10 and 23 of the menstrual cycle. *Numbers* represent the mass/charge ratio marked by a line. m/z values within 0.3% of each other represent the same peak
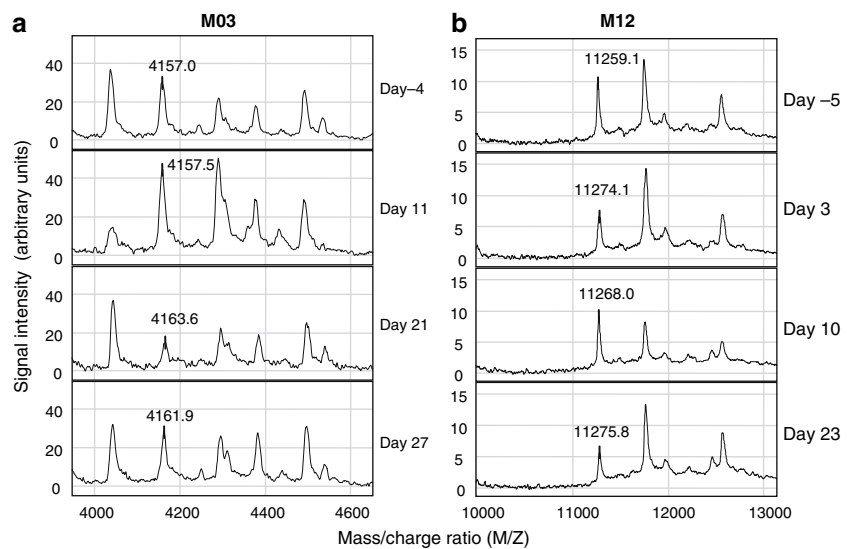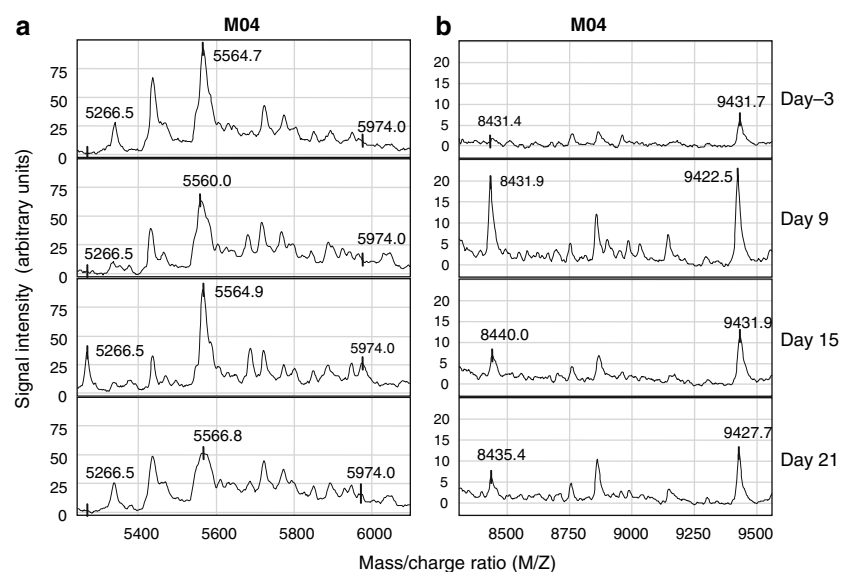

Fig. 2 Statistically significant peaks in healthy volunteer M04 on the IMAC30 array. a The statistically significant peaks at 5,267, 5,563, 5,975 Da and **b** at 8,432 and 9,427 Da from healthy volunteer M04 on days -3, 9, 15 and 21 of the menstrual cycle. *Numbers* represent the mass/charge ratio marked by a line. m/z values within 0.3% of each other represent the same peak

Importantly when the spectra from different volunteers were compared, there was no overlap of the statistically significant peaks between patients. Furthermore, peaks which were statistically significant in one patient are present but not statistically significant in other patients.

Discussion

This study shows that there is only a small amount of variation in proteomic profile across the 4 weeks of the menstrual cycle for each healthy volunteer. In addition, the peaks that varied across the menstrual cycle were different from one healthy volunteer to another. Moreover, these variations are small compared to the

large variation in proteomic profile between healthy volunteers. We could conclude that the variation in peak intensity between weeks of the menstrual cycle is therefore insignificant.

There were several limitations in this study, which affected the data analyses. The low numbers of healthy volunteers recruited resulted in a low level of statistical significance. Recruitment was difficult due to the restricted inclusion criteria, in particular current oral contraceptive use ruled out many potential recruits. The design of the study required regular and frequent attendance for 2 months, which was difficult to achieve in volunteers. Although small variations are present in the samples in the same healthy volunteer, it is not possible to say whether these were due to the menstrual cycle. Data would need to be collected over

many cycles in the same healthy volunteer to check whether the variation is consistent.

A previous study has examined the cellular characteristics [10] and PSA levels of NAF over the menstrual cycle in 14 healthy volunteers [10, 11]. Fourteen women underwent weekly nipple aspiration for two menstrual cycles. No significant variation was found in cell number or cell type during the menstrual cycle. NAF PSA levels varied between samples taken from the same breast at different stages of the menstrual cycle but no cyclical change in NAF PSA levels throughout the menstrual cycle were observed. Research has also been carried out to examine variation in NAF constituents over time. A study has examined oestradiol levels in NAF at fixed points in the menstrual cycle over 15 months. A high stability of the concentration of oestradiol was found over time suggesting environmental factors had little immediate effect on NAF oestradiol levels. Levels of cathepsin D, epidermal growth factor and interleukin-6 in NAF were consistent throughout the menstrual cycle [12].

The study presented here is the first to examine the NAF proteome during the menstrual cycle. The data obtained demonstrated that there was no cyclical variation in NAF proteomic profile in any individual healthy volunteer. Moreover, greater interhealthy volunteer variation than intrahealthy volunteer variation was observed. In conclusion, this study suggests that the NAF proteomic profile does not vary substantially during the menstrual cycle and that therefore it is valid to compare NAF profiles from pre-menopausal women which have been taken at different phases of the menstrual cycle.

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