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Proteomic profiling of nipple aspirate fluid (NAF): Exploring the complementarity of different peptide fractionation strategies

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ABSTRACT

NAF is a breast fluid that is closely related to the tumor microenvironment and a valuable sample for studying breast cancer. To perform an in-depth proteomic analysis of this sample, aliquots of a single NAF digest were analyzed by the following peptide-centric fractionation strategies: a) 30-cm reversed-phase (RP) column on-line with an LTQ-Orbitrap XL; b) off-line strong cation-exchange (SCX) column; and c) pI-based OFFGEL fractionation. All fractions from approaches (b) and (c) were further analyzed on a 10-cm RP column hyphenated to the same mass spectrometer. The RP-30 cm, SCX/RP-10 cm, and OFFGEL/RP-10 cm approaches identified 1676, 2930, and 3240 peptides, which corresponded to 193, 390 and 528 proteins, respectively. In our cumulative dataset, 4466 distinct NAF peptides corresponded to a total of 557 proteins, of which only 34% were identified by all three approaches. No exclusive protein identification was associated to the RP-30 cm approach, while SCX/RP-10 cm and OFFGEL/RP-10 cm contributed to 28 and 166 exclusive identifications, respectively. Each approach provided additional information related to energy metabolism (fermentation process/carbohydrate biosynthesis). In conclusion, the pre-fractionation platforms used were complementary for the comprehensive characterization of NAF and our work provides methodological information for future quantitative cancer-related NAF sample studies.

Biological significance
High-resolution peptide separation is a *sine qua non* condition for achieving extensive proteome coverage. Various techniques have been employed to improve peptide fractionation prior to LC-MS/MS, thus allowing a comprehensive characterization of complex biological samples. Although fractionation efficiency is very sample-dependent, this issue is commonly overlooked, and a "cookbook" approach is routinely used during this critical step. The present study provides a systematic comparison of analytical information needed

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for the successful large-scale differential proteomic analysis of NAF samples from breast cancer patients, a precious and volume-limited biological sample. It reinforces the importance of optimizing sample-specific fractionation protocols for information retrieval from mass spectrometric analysis.

1. Introduction

Nipple aspirate fluid (NAF) is a fluid secreted by the epithelial cells of the mammary ductal and lobular system, and it contains a set of specific breast tissue proteins. In breast cancer cases, these proteins are closely related to the microenvironment of the development and progression of breast malignant tumors [1]. It is expected that some diseases (*e.g.*, cancer) can be indirectly studied by monitoring variations in the proteomic profiles of body fluids [2]. In one of our previous works, NAF samples from both breasts of seven Brazilian patients with unilateral breast cancer were systematically compared using gel-based methodologies (zymography, SDS-PAGE and 2D-DIGE). We detected important individual NAF protein expression variability that could be correlated with the aggressiveness of the disease [3].

Changes in the NAF composition that may be related to breast cancer are still poorly understood [4] and a comprehensive description of the proteins that make up the NAF secretome from healthy and diseased person is the first step in better understanding this disease through proteomics. An optimized analytical strategy facilitates deeper digging into the proteomes of complex biological samples. The widely adopted approach is to fractionate the sample prior to mass spectrometry analysis. Protein and/or peptide separation methods, such as strong cation-exchange (SCX) chromatography, isoelectric focusing (IEF) and gel electrophoresis (SDS-PAGE), are the most commonly adopted pre-fractionation strategies for proteomic investigations in the biomedical sciences [5]. More recently, with improved chromatographic separation and the novel parallelized quadrupole/linear ion trap/Orbitrap tribrid mass spectrometer [6], the single-shot proteomics mode has emerged as a very fast and promising method for the in-depth analysis of complex proteomes. One of the most striking examples of this powerful approach was the detection of 3977 yeast proteins during a 70-min analysis time [7]. Unfortunately, due to the high equipment costs, the single-shot proteomics strategy is still far from reality in most academic laboratories.

Several protein classes have been detected in diseased and non-diseased NAF conditions using one- and two-dimensional electrophoresis (1D-PAGE and 2D-PAGE), liquid chromatography, and mass spectrometry (MALDI/SELDI-TOF) approaches [8–13]. However, only a few cancer-related proteins have been found with these techniques. Moreover, the results were not consistent among different studies. The results varied according to the sample preparation protocol, the type of array chips used and the MS peak analysis strategy that was applied in the SELDI-TOF studies [14]. When comparing NAF samples from diseased and healthy donors, several differential mass spectral peaks were characterized by SELDI-TOF, although no protein identification was achieved [10,15,16].

In a typical shotgun proteomics experiment, orthogonal unidimensional liquid chromatography is used as a first fractionation step prior to the analysis by RP liquid chromatography-tandem mass spectrometry (LC-MS/MS) [17]. Each fraction represents a more simplified peptide mixture, thus increasing the chance for identification of low-abundance proteins by MS/MS [18]. Only one descriptive shotgun study of NAF has been published so far [19], involving NAF samples that were collected from subjects submitted to breast biopsies. The "cancer group" was composed of two NAF samples that were collected from breasts diagnosed with cancer and of one sample from the contralateral healthy breast of a cancer patient. The "control group" was composed of three NAF samples that were collected from breasts that had biopsies negatively diagnosed for cancer. Each pair of "control" and "cancer" NAF was analyzed by one of the following distinct fractionation conditions prior to nLC-MS/MS: size exclusion chromatography of intact NAF proteins followed by in-solution trypsin digestion; acetone precipitation of NAF proteins followed by trypsin digestion and peptide fractionation by SCX; and strong anion exchange (SAX) of NAF proteins followed by trypsin digestion and SCX fractionation. Using these methodologies, the authors were able to identify 854 unique proteins, the largest inventory of NAF proteins reported so far. It is important to stress that because different samples were analyzed by different methodologies, it is challenging to draw conclusions on the complementarity of the approaches employed for the NAF profiling.

The aim of this work was the in-depth characterization of the breast proximal fluid (NAF) by shotgun analysis, exploring the complementarity of different peptide-centric fractionation strategies. For this purpose, three aliquots of a tryptic digest of a single NAF sample (collected from a subject's breast that had a benign breast disease) were independently fractionated by: (a) a 30-cm RP column hyphenated to an LTQ-Orbitrap XL; (b) an off-line SCX column eluted with a linear salt gradient (12 fractions) or (c) a pI-based OFFGEL fractionation (pH 3–10, 12 fractions); all fractions from approaches (b) and (c) were further analyzed on a 10-cm RP column that was coupled on-line to the same mass spectrometer used in (a).

2. Materials and methods

2.1. NAF collection

A NAF sample collected from a single healthy breast of a woman with unilateral fibroadenoma benign proliferative lesion was used for all analyses. NAF collection and protein quantification were performed as previously described [3]. No protease inhibitor cocktail was added to the sample, which was collected after approval from the Fernandes Figueira Institute Research Ethics Committee (license 0083/10), following written informed consent. The woman had not breastfed for the last two years, had not taken exogenous hormones for the previous six months, had no previous breast surgery, and