The follicular microenvironment as a predictor of pregnancy: MALDI-TOF MS lipid profile in cumulus cells

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Abstract

Purpose This research proposed to study the changes in lipid composition in cumulus cells (CCs) from women who achieved pregnancy compared with women who did not, after in vitro fertilization treatment. This approach has the potential to provide novel information on the lipid metabolism of the CCs and as an additional method to predict pregnancy.

Method Fifty-four samples from couples with tubal and male factor infertility and where the female partner was age 35 or younger were divided in two groups according to their level of hCG 14 days after embryo transfer as follows: (1) 23 samples in pregnant group and (2) 31 samples in non-pregnant group. Lipid extraction was performed by the Bligh-Dyer protocol, and lipid profiles were obtained by MALDI-TOF MS. Mass spectra data were processed with MassLynx, and statistical analysis was performed using MarkerLynx extended statistic. OPLS-DA model was built. **Results** S-plot Analysis revealed three ions as potential markers in the pregnant group, and five ions in the non-pregnant group. These ions were identified in the human metabolome database (HMDB) as phosphatidylcholine in the pregnant group and as phosphatidylethanolamine,

phosphatidylserine and phosphatidylinositol species in the non-pregnant group. These lipids might be involved in cell proliferation and differentiation, apoptosis and GAP junction regulation.

Conclusion We conclude that MALDI-TOF MS can be used as an informative and fast analytical strategy to obtain and study the lipid profile of cumulus cells and can potentially be used as a supporting tool to predict pregnancy based on the metabolic state of the CCs.

Keywords Cumulus cell · Lipid profile · MALDI-TOF MS · Pregnancy outcome

Introduction

Aspects of embryo morphology have been used to predict pregnancy during in vitro fertilization (IVF) treatment. These include the number of blastomeres in a given stage of development, the regularity of cell division and the degree of fragmentation [2]. Although embryo morphology has been correlated with implantation potential [6], selecting embryos with the appropriate morphological appearance alone is not sufficient to guarantee embryo viability and improve the chances of pregnancy. Several methods, such as removing the polar body [34] and one blastomere from the embryo on the third day of culture [28], were proposed to evaluate oocyte and embryo development, although it is not known what impact these techniques have on embryonic development. Therefore, new non-invasive approaches in oocyte and embryo metabolomics appear to be an alternative for gamete selection and have been proposed for assessing the potential of oocytes and embryos using embryo culture media, follicular fluid and cells [6].

Capsule Lipid profile by MALDI-TOF MS in cumulus cells as a predictor of pregnancy.

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The ability to achieve good embryo development is reported to be dependent on oocyte competence, which is acquired during folliculogenesis [40]. Thus, follicle maturation is crucial for an oocyte to become a good quality embryo and consequently achieve a successful pregnancy [19].

During follicular development, the granulosa cells differentiate into two layers; the first layer, which is composed of theca cells, coats the follicular antrum and is responsible for estrogen production and follicular rupture, and the second layer, which consists of cumulus cell (CCs), involves the oocyte. The function of these cells is partially regulated by oocyte factors, and therefore CCs assist with oocyte maturation and further development [14].

The interaction of the oocyte and the CCs occurs at gap junctions [41], through which nutrients and maturation-enabling factors are provided and where the exchange of metabolites and ions between the oocyte and the CCs is facilitated [13]. Therefore, reciprocal and optimal exchange between the oocyte and the CCs is essential for the acquisition of oocyte competence. It has been hypothesized that studying these cells can elucidate the physiological importance of these processes and may lead to optimization of IVF [41]; the lipid composition of the CCs may reflect and determine oocyte maturation, fertilization and early embryo development potential.

With the rise of new technologies based on mass spectrometry such as the *-omics* approaches (proteomics, metabolomics, lipidomics), which provide detailed molecular information, novel analytical tools can be developed and applied to IVF to discover new biomarkers to predict pregnancy [21]. Indeed, the lipidomic approach based on mass spectrometry coupled with a lipid database search can investigate the potential interactions of lipid signaling and their impact in modulating physiology [5].

Lipids are essential cellular constituents. They have many functions within cells and regulate several biological processes. Lipids are the building blocks of bilayer membranes and regulate key cellular functions by acting as signaling molecules and precursors for second messengers and by regulating signal transduction pathways, cell proliferation and apoptosis [20].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been successfully employed as a tool for the analysis of lipid composition because it provides results within a few minutes with a minimal degree of analyte fragmentation, which allows for the analysis of highly complex structures [37].

The introduction of more complex data treatments from a mathematical and statistical standpoint has been carried out in multivariate analysis. This allows the analysis of multiple variables at the same time and draws a smaller number of variables, which allow for the visualization of data [33]. The two most common multivariate analyses are the principal

component analysis (PCA), which is an unsupervised test, and the orthogonal partial least square discriminate analysis (OPLS-DA), which is a supervised test employed to build a predictive model [23].

Lipid profile analysis of CCs, which are usually discarded during oocyte retrieval, may indirectly reflect the competence of the oocyte. Because of the importance of CCs for the oocyte viability, the present study compared the lipid MALDI-TOF MS profile of CCs from women who did and did not achieve pregnancy following IVF treatment. The goal of the study was to investigate the metabolism of the CCs and to evaluate whether changes in the lipid profile of the CCs can be used as a prognostic tool to identify lipids that are indicative of oocyte competence. To our knowledge, this is the first time that MALDI-TOF MS has been used to study the lipid composition of CCs, which has a promising application for assisted reproductive technologies (ART).

Materials and methods

Study group

An observational case-control study was carried out among patients seeking IVF treatment at the Human Reproduction Section of Sao Paulo Hospital. This study received approval from the Institutional Review Board of the São Paulo Federal University Research Ethics Committee (protocol: 1299/10).

Samples of CCs were obtained from 54 women up to 35 years old without endometriosis, polycystic ovarian syndrome or ovarian dysfunction, who underwent in vitro fertilization (IVF) by intracytoplasmic sperm injection (ICSI) after referral to the IVF program at São Paulo Federal University. To ensure that male factor infertility did not affect the model, sperm with less than 3 million sperm ml^{-1} and above 4 % strict morphology according to the Kruger classification [24] were excluded. Patients were subdivided into two groups. The first group consisted of 23 samples from patients who achieved pregnancy (age 30.61 ± 3.88 years, mean $\pm$ SD). The second group consisted of 31 samples from women referred to the IVF program who did not achieve pregnancy in that treatment cycle (age 30.97 ± 3.48 years, mean $\pm$ SD).

All patients underwent IVF because of tubal factor female infertility and/or mild male factor infertility. For both groups, only couples in which the female partner was 35 years or younger and had a serum follicle-stimulating hormone (FSH) level of between 3 and 9 $\mu\text{g/ml}$ on Day 3 of the menstrual cycle previous to the treatment cycle were included.

All women received a similar ovarian stimulation protocol for IVF. Controlled ovarian stimulation was performed with the use of exogenous recombinant gonadotropins (225 IU/day of Gonal-F, Merck-Serono, Darmstadt, Germany)

starting on cycle day 2. When the leading follicle reached 14 mm in diameter, endogenous LH release was suppressed with the use of a GnRH antagonist analog (Cetrorelix—Cetrotide; Merk-Serono) until the day of hCG administration. When the leading follicle reached 17 mm in diameter, a total dose of 250 µg of hCG was administered. Ultrasound-guided transvaginal oocyte retrieval was performed 36 h after hCG administration. For all patients, the excess of CCs were collected prior to fertilization, after follicle pick-up from the group of oocytes present in both ovaries. Removal of excess of cumulus cells is routinely performed prior to oocytes incubation in our laboratory. This procedure is only performed in oocytes with expanded cumulus cells and in patients who will be submitted to in vitro fertilization by ICSI. Thus, the samples of cumulus cells in the present study were collected prior to fertilization, which was performed by ICSI in mature oocytes (MII). Samples of CCs were stored in microtubes at −20 °C until lipid extraction.

Lipid extraction

Lipids were extracted based on the protocol by Bligh and Dyer (Bligh et al. 1959) with some modifications. Briefly, 50 µL of distilled water was placed in a microtube with the CCs, and they were mixed with a pipette to break the cells by osmotic lysis. The next step was to add 125 µL of chloroform and 250 µL of methanol. The mixture was homogenized and vortexed for 1 min. The polar and apolar phases were separated by the addition of 100 µL of water and 125 mL of chloroform. The mixture was vortexed for 1 min and centrifuged at 3,000× g for 1 min. The lower phase containing the lipids was recovered and transferred to a clean microtube, which was left open at room temperature for 1 h until the solvent evaporated.

MS data acquisition

A volume of 10 µL of chloroform was added to each sample to dissolve the lipids contained in the microtube. For MALDI-TOF MS analysis, 2 µL of each sample were deposited on the MALDI target plate and covered with 1 µL of 2,5-dihydroxybenzoic acid (DHB 0.5 M) dissolved in 90 % methanol. Mass spectra were acquired in the positive ion mode using a Q-ToF Premier (Synapt HDMS) mass spectrometer (Waters, Manchester, UK) equipped with a 200 Hz solid-state laser in the m/z 700–1,200 range in the reflectron mode. Typical operating conditions were: laser energy 250 a.u., sample plate 20 V and the *Trap* and *Transfer* collision energies were 6 and 4 V, respectively (QTOF-MS mode).

Data analysis

The mass spectra of each sample were accumulated using MarkerLynx 4.1 software (Waters, Manchester, UK) and

exported for principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA) by MarkerLynxTM XS (Waters, Manchester, UK). The method parameters were as follows: mass tolerance = 0.5 Da, baseline noise = 50 and intensity threshold (count) = 1,000 with deisotope data.

The S-plot of the OPLS-DA analysis provided a list of ions responsible for the differences between the groups, and the lipid subclasses of these ions were searched in the Human Molecular Database (HMDB) (<http://www.hmdb.ca>). A mass tolerance of 0.1 Da was adopted. Mass error in ppm was calculated for all of the ions found and was considered only when the mass error was ≤50 ppm.

Results

A total of 54 samples of CCs were obtained from women undergoing IVF, of which 23 samples were from woman who achieved pregnancy and 31 samples were from woman who did not achieve pregnancy. Clinical data including the

Table 1 Clinical characteristics of patients from the pregnant group and the non-pregnant group

	Pregnant group	Non-pregnant group	<i>P</i> -value
Age (years)			
Mean; SD	30.61; 3.88	30.97; 3.48	0.72
95 % CI	26.73–34.49	27.49–34.45	
FSH (µgml ^{−1})			
Mean; SD	6.17; 1.89	6.96; 2.20	0.17
95 % CI	4.29–8.06	4.77–9.16	
LH (µgml ^{−1})			
Mean; SD	4.39; 1.77	4.72; 2.07	0.54
95 % CI	2.61–6.16	2.65–6.78	
Follicles (n)			
Mean; SD	20.39; 12.58	14.29; 6.59	0.02
95 % CI	7.81–32.97	7.70–20.88	
Oocytes (n)			
Mean; SD	12.70; 8.11	8.10; 4.39	0.01
95 % CI	4.59–20.81	3.70–12.49	
Recovery rate (%)			
Mean; SD	0.64; 0.21	0.57; 0.17	0.22
95 % CI	0.43–0.85	0.40–0.75	
MI oocytes (%)			
Mean; SD	0.66; 0.24	0.82; 0.19	0.009
95 % CI	0.42–0.91	0.63–1.01	
Fertilization rate (%)			
Mean; SD	0.78; 0.24	0.70; 0.27	0.22
95 % CI	0.54–1.02	0.43–0.97	

Bold *P*-values indicate statistical significance

Fig. 1 Typical MALDI-MS data for the pregnant and non-pregnant groups. The y-axis shows relative abundances whereas the x-axis shows m/z values

patients' number of follicles, the number of oocytes retrieved and the rate of mature oocytes (MII) are presented in Table 1, and the representative spectra of each group are shown in Fig. 1.

Multivariate statistical analyses, such as PCA and OPLS-DA, were carried out on preprocessed MALDI-TOF-MS data, including the relative ion intensities and m/z values, to understand the difference in lipid profiles. The PCA 2D plot of the samples from the pregnant and non-pregnant groups showed that the groups could be differentiated (Fig. 2). Because individual variation, PCA was not satisfactorily separate the experimental groups. The explained

variance of each principal component is shown in Table 2. The OPLS-DA (Fig. 3a) was also performed, and the pregnant and non-pregnant groups were clearly separated, demonstrating that the OPLS-DA was more effective for predicting pregnancy using the CCs lipid profile.

To detect the differential lipids related to the separation of the pregnant and non-pregnant groups, three and five ions, respectively, with the highest covariance between groups were selected from the S-plot (Fig. 3b) of the OPLS-DA as potential biomarker candidates. From the three ions selected from the CCs samples of the pregnant group, and only one was identified as phosphatidylcholine. In the non-

Fig. 2 PCA 2D shows the variance between groups by scores of PC1 x PC2. The black points show the pregnant group by individual PCs; the red points show the non-pregnant groups

Table 2 Percentage of variance explained by principal component analysis (PCA)

Number of component	Individual variance explained (%)	Cumulative variance explained (%)
1	23.38 %	23.38 %
2	12.32 %	35.70 %
3	9.58 %	45.27 %
4	6.56 %	51.83 %
5	5.63 %	57.47 %
6	5.25 %	62.72 %
7	3.67 %	66.39 %
8	3.31 %	69.70 %
9	2.79 %	72.50 %

pregnant group, of the five ions selected, four were identified as phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI). Therefore, all lipids identified in this study belong to the phospholipid subclass. Ions (m/z), mass error (ppm) and the lipid subclasses identified are shown in Table 3.

Discussion

Aspects of embryo morphology have prognostic importance for pregnancy following ART [18,45]. Given the role of CCs in oocyte maturation support, that they actively influence the oocyte's acquisition of developmental competence, and that many cumulus function are regulated by the oocyte [14], the chemical analysis of these cells may be a promising approach for the non-invasive assessment of oocyte competence, embryo development potential and future pregnancy. Several groups have been searching for new markers of oocyte competence and pregnancy outcome in these cells [1,3]. However, to our knowledge, this work is the first time

that analysis of the lipid profile of CCs by MALDI-TOF MS has been studied.

The clinical data analysis revealed that the number of follicles and number of oocytes were higher in pregnant women than in non-pregnant women, which did not influence the retrieval rate. However, the MII rate was higher in the group of women who did not achieve pregnancy. The increase in the number of follicles and the number of oocytes retrieved in the pregnant group only suggests that these women had higher follicular recruitment compared to the group that did not achieve pregnancy, but it does not necessarily indicate that all of the oocytes were completely matured. On the other hand, the analysis of clinical data has shown that the non-pregnant group had a higher average rate of retrieval of mature oocytes. Our findings regarding the lipid profile in the group with unsuccessful treatment indicate that the retrieval of mature oocytes in this group may have been inadequate. Even though we collected only CCs expanded, eventually we might have been collected CCs from immature oocytes, like oocytes in metaphase I (MI), which also show expanded DC. However, were not observed statistical difference in percentage of mature and immature oocytes (Table 1) indicates that the oocyte maturity was has not been influence in the results of lipid profile.

The intensity of ions found were above the set detection limit; however, the different ions observed between the groups were not the most intense on the spectrum (Fig. 1). Therefore, it would not be possible differentiate them without the use of statistical tools.

Phosphatidylcholine, identified as ions with m/z 886.6428 in the pregnant group, is the lipid subclass that is most abundant in cell membranes, and lipids of this class constitute 40–50 % of total phospholipids. PC roles have been well established in differentiation and cell proliferation [43]. CCs undergo proliferation before the LH surge due to the action of growth factors secreted by the oocyte to regulate the function of the CCs [17]. During the pre-ovulatory

Fig. 3 OPLS-DA 3D Plot (a) shows scores of individual samples x CP1 x CP2 of pregnant (black squares) and non-pregnant (red squares) groups and the S-Plot (b). Lipids denoted by the red square could be selected as the lipids important for the separation of the two groups (Table 3)

Mass (m/z)	Group	Chemical Formula	Lipid subclass	Adducts	Mass error (ppm)
750.4191	NP	C38H66NO10P	PS(18:3(9Z,12Z,15Z)/14:1(9Z)) PS(14:1(9Z)/18:3(9Z,12Z,15Z))	M+Na [1+] M+Na [1+]	16.67
807.4818	NP	C41H75O13P	PI(16:2(9Z,12Z)/16:0) PI(16:1(9Z)/16:1(9Z)) PI(16:0/16:2(9Z,12Z))	M+H [1+] M+H [1+] M+H [1+]	24.72
806.4842	NP	C42H74NO10P	PS(20:4(5Z,8Z,11Z,14Z)/16:0) PS(20:3(8Z,11Z,14Z)/16:1(9Z)) PS(18:3(9Z,12Z,15Z)/18:1(9Z)) PS(18:2(9Z,12Z)/18:2(9Z,12Z)) PS(18:1(9Z)/18:3(9Z,12Z,15Z)) PS(16:1(9Z)/20:3(8Z,11Z,14Z)) PS(16:0/20:4(5Z,8Z,11Z,14Z))	M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+]	12.41
822.4423	NP	C45H70NO8P	PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/18:4(6Z,9Z,12Z,15Z)) PE(20:5(5Z,8Z,11Z,14Z,17Z)/20:5(5Z,8Z,11Z,14Z,17Z)) PE(18:4(6Z,9Z,12Z,15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	M+K [1+] M+K [1+] M+K [1+]	0.001
1041.54	NP	UNIDENTIFIED			>50
886.6428	P	C52H88NO8P	PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/22:2(13Z,16Z)) PC(22:4(7Z,10Z,13Z,16Z)/22:4(7Z,10Z,13Z,16Z)) PC(22:2(13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	M+H [1+] M+H [1+] M+H [1+]	12.18
		C50H90NO8P	PC(20:0/22:5(7Z,10Z,13Z,16Z,19Z)) PC(20:0/22:5(4Z,7Z,10Z,13Z,16Z)) PC(18:4(6Z,9Z,12Z,15Z)/24:1(15Z)) PC(24:1(15Z)/18:4(6Z,9Z,12Z,15Z)) PC(22:5(7Z,10Z,13Z,16Z,19Z)/20:0) PC(22:5(4Z,7Z,10Z,13Z,16Z)/20:0) PC(22:4(7Z,10Z,13Z,16Z)/20:1(11Z)) PC(22:2(13Z,16Z)/20:3(8Z,11Z,14Z)) PC(22:2(13Z,16Z)/20:3(5Z,8Z,11Z)) PC(22:1(13Z)/20:4(8Z,11Z,14Z,17Z)) PC(22:1(13Z)/20:4(5Z,8Z,11Z,14Z)) PC(22:0/20:5(5Z,8Z,11Z,14Z,17Z)) PC(20:5(5Z,8Z,11Z,14Z,17Z)/22:0) PC(20:4(8Z,11Z,14Z,17Z)/22:1(13Z)) PC(20:4(5Z,8Z,11Z,14Z)/22:1(13Z)) PC(20:3(8Z,11Z,14Z)/22:2(13Z,16Z)) PC(20:3(5Z,8Z,11Z)/22:2(13Z,16Z)) PC(20:1(11Z)/22:4(7Z,10Z,13Z,16Z))	M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+] M+Na [1+]	14.94
858.9356	P	UNIDENTIFIED			>50
917.06	P	UNIDENTIFIED			>50

Fig. 4 Metabolic correlation between phosphatidylserine (PS) and phosphatidylethanolamine (PE), found in the non-pregnant group, and phosphatidylcholine (PC), found in the pregnant group. *PSS1* PS synthase-1; *PSS2* PS synthase-2; *PSD* phosphatidylserine decarboxylase; *PEMT* phosphatidylethanolamine N-methyltransferase; *ET* ethanolamine transferase; *EK* ethanolamine kinase; *CPT* phosphoethanolamine cytidyltransferase; *EPT* ethanolamine phosphotransferase; *CT* ethanolamine transferase; *CK* choline kinase

Phosphatidylethanolamine (PE) is the second most abundant component of phospholipid membranes, constituting 20–50 % of total phospholipid, and was identified in the non-pregnant group as the ion with m/z 822.4423. PE performs several biological functions in addition to being a primary constituent of the inner leaflet of the plasma membrane. Among the biological functions described in previous studies of PE redistribution and translocation between the bilayer membranes, there are two principal functions of PE that are of interest in our study. First, PE is redistributed and transiently externalized during mitotic cell division [12], which may occur during proliferation of the CCs. PE is also redistributed and exposed to the extracellular environment that signals cell death when the membrane asymmetry is compromised [32]. Li et al. described that the decrease in the ratio of PC to PE was associated with a reduction in membrane integrity (Caballero et al.2010;[25]). However, a more recent study attributed the loss of membrane integrity to PC deficiency alone [31]. Otherwise, low PC intensity and high PE intensity in our study may indicate that the CC in non-pregnant women could be undergoing apoptosis. Nevertheless, more structural and functional studies of PE are needed to understand the mechanism that is occurring.

Phosphatidylserine (PS) is also observed in the non-pregnant group at relatively low levels and constitutes 2–10 % of the total phospholipid membrane. It was identified through the two ions found in this group with m/z 750.4191 and 806.4842. In cells, PS is located exclusively in the intracellular environment (Caballero et al.), which plays an important role because many intracellular proteins, such as protein kinases, require PS for proper activation and/or location [7,25,31]. Moreover, PS has also been described

as a marker of apoptosis [4,15,16]. In contrast, other studies have been suggested that an increase in PS protects cells against apoptosis [22,30,36]. The postulated mechanism is that an increase in PS synthesis increases translocation of Raf-1, a kinase involved in apoptosis, to the membrane. In both mechanisms, PS is exposed on the cell surface and/or in the extracellular environment. Regardless of whether PS was increased in the non-pregnant group as a marker of apoptosis or as a marker of anti-apoptosis mechanisms, it is clear that these CCs were damaged, and their altered roles may leads to injury during oogenesis in these patients.

Finally, PI was identified in the non-pregnant group (m/z 807.4818) and is found relatively less often in the phospholipid cell membranes of mammals [42]. PI is a glycerophospholipid component in eukaryotic cells [36]. PI and its derivatives have been described as precursors for cellular signaling molecules [4] and in the biosynthesis of the glycerophosphatidylinositol anchor [30,36], besides being a structural component of the membrane [15]. PI and its phosphorylated forms, together with a phosphoinositide-specific phospholipase C (PI-PLC), generate diacylglycerol (DAG) [22], which is another lipid subclass. This pathway was described as being responsible for stimulating protein kinase (PK) activation, including mitogen-activated kinase protein (MAPK), which has been previously reported as an attendee in the regulation of oocyte meiotic resumption, in which it may act directly or indirectly [9]. Recent studies have shown that MAPK is required in gonadotropin-induced meiotic resumption [35,38,39,44]. Other studies have also established that MAPK as well as protein kinase A (PKA) interrupts the communication between the CCs and the oocyte, thus decreasing the cyclic adenosine

monophosphate (cAMP) level in the female gamete, which initiates its meiotic resumption [26,29,35]. Therefore, we propose that the high PI intensity in the NP group indicates that the degradation of DAG has been not occurring or that it has been occurring at low activity, due to the reduced efficiency of meiotic resumption in this group.

Conclusion

Our study compared the lipid profile of cumulus cells from women who achieved pregnancy and woman who did not achieve pregnancy following IVF treatment by MALDI-TOF MS. These results suggest, through the analysis of lipid subclasses, that it is possible to predict pregnancy occurrence or failure and thus contributes to a better understanding of the putative mechanisms involved in oocyte development. Our results provide the possibility for studies of the structural characterization of these molecules and also provide opportunities to investigate the possible relations between gene and protein expression and the lipids subclasses observed in this pioneering study.

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