



Proteomics, Metabolomics and Lipidomics in Reproductive Biotechnologies: The MS Solutions

Christina Ramires Ferreira¹, Edson Guimarães Lo Turco², Sergio Adriano Saraiva¹, Ricardo Pimenta Bertolla², Felipe Perecin³, Gustavo Henrique Martins Ferreira Souza⁴, Michael Murgu⁴, Jerusa Simone Garcia⁵, Sylvia Sanches Cortezzi⁶, Flávio Vieira Meirelles³, Clécio Fernando Klitzke¹, Elaine Cristina Cabral^{1,7}, Maria Angélica Miglino⁸, Patricia Marafon Porciuncula^{1,2}, Claudia Lima Verde Leal³, Edson Borges Jr.⁹, Daniele dos Santos Martins³, Carlos Eduardo Ambrósio³, Fabio D´Alexandri¹, Lawrence Charles Smith^{3,10} & Marcos Nogueira Eberlin¹

ABSTRACT

Background : A broader view of living systems complexity is bringing important contributions to biological sciences, since the genome expression is affected by other classes of molecules, which in their turn interact themselves in cellular metabolic pathways and biochemical networks. This level of information has been made possible by the emergence of the omic strategies, such as proteomics, metabolomics and lipidomics, that are mainly based on mass spectrometry (MS) platforms. MS has presented an incredible development over the last years, evolving to a powerful and universal analytical technique. Its ability to analyze proteins and small molecules such as lipids, sugars and metabolites at the structural level, with sensitivity and speed inconceivable a few years ago, is the major driving force in the omic fields. The development of electrospray and matrix-assisted laser desorption/ionization (MALDI) ionization techniques has decisively contributed to the many applications of this technology nowadays. Herein, we present and discuss omic concepts and strategies, as well as detail basic principles of MS. Applications and future perspectives of these approaches are focused in the reproductive medicine area.

Review : The omic technologies propose global characterization of specific classes of target biomolecules of cellular systems as a strategy to achieve comprehensive understanding of biological functions. The genomics, aimed at performing the entire genetic sequencing of organisms, represented the seminal step towards the understanding of the complex logic that orchestrates the function of all organisms or the defects leading to diseases. But to express the phenotype, information needs to flow from DNA via carrier biomolecules through processes that are being addressed by new omic sciences such as the transcriptomics, proteomics, metabolomics, glycomics, lipidomics, and fluxomics. Mass spectrometry (MS) is nowadays the most powerful technique for the structural characterization of biomolecules, and has therefore become the central technique for the omic sciences. Using revolutionary ionization techniques such as electrospray (ESI) and matrix-assisted laser desorption ionization (MALDI), a wide range of biomolecules such as peptides, proteins, lipids and sugars are efficiently transferred in intact ionized forms to the gas phase for MS analysis. The development of ESI-MS and MALDI-MS has been awarded the Nobel Prize for Chemistry in 2002, rocketing the application of MS in the omic sciences. More recently, ambient ionization MS techniques, such as desorption electrospray ionization (DESI) and easy ambient sonic-spray ionization (EASI), have been developed for ionization in the open atmosphere, in a workup free and high throughput fashion directly from sample in their original environments. For the more complex samples, the coupling with separation techniques such as liquid chromatography (LC) as well as the use of tandem MS (LC-MS/MS) has allowed comprehensive mixture characterization of major biomolecules.

Conclusion: This manuscript describes recent advances of MS in the proteomics, metabolomics and lipidomics for biological sciences, and points out the relevant contributions that MS is likely to bring to fundamental and applied research in human and animal embryo biotechnologies.

Keywords: mass spectrometry, proteomics, lipidomics, metabolomics, embryo, biotechnology.

¹ThoMSon Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas - UNICAMP, 13083-970, Campinas, SP, Brazil. ²Division of Urology, Department of Surgery, São Paulo Federal University - UNIFESP/EPM, São Paulo, SP, Brazil. ³Faculty of Animal Sciences and Food Engineering - FZEA, University of São Paulo (USP), Pirassununga, SP, Brazil. ⁴Waters Technologies do Brasil, Baurer-SP, Brazil. ⁵Department of Exact Sciences, Alfenas Federal University, Alfenas, MG, Brazil. ⁶Sapientiae Institute, São Paulo, SP, Brazil. ⁷Chemistry Institute, São Paulo, SP, Brazil. ⁸Medicine Veterinary University - USP, São Paulo, SP, Brazil. ⁹Fertility - Assisted Fertilization Center São Paulo, SP, Brazil. ¹⁰Université de Montréal, Saint-Hyacinthe, Québec, Canada. CORRESPONDENCE: C.R. Ferreira [christina@iqm.unicamp.br - TEL: +55 (19) 35213049].

I. INTRODUCTION

II. MOLECULAR STRUCTURAL INFORMATION FROM YOUR SAMPLE: “CALL MASS SPECTROMETRY!”

III. MS IN “THE QUEST” FOR PROTEIN BIOMARKERS

IV. METABOLOME: THE “INFORMATION SOUP”

V. MAKING LIPIDS FLY BY MS

VI. “REAL-WORLD” PERSPECTIVES OF OMIC APPLICATIONS ON EMBRYO TECHNOLOGIES

VII. CONCLUSIONS

I. INTRODUCTION

Since the genomics revolution, a number of scientific efforts have also allowed the growth of our knowledge regarding the characterization, function and interaction of other key biomolecules for the cell such as DNA transcripts, proteins, and metabolites. These efforts gave birth to other omic fields known now as transcriptomics, proteomics, and metabolomics (Figure 1). Currently, subsets of metabolomics are evolving such as lipidomics, glycomics, and fluxomics with the ultimate goal of integrating the omics picture by means of the interactome of genes, transcripts, proteins, and metabolites representative of the cellular function complexity [12].

Proteomics is generally defined as the identification and quantification of all the expressed proteins of a biological sample, such as cells or embryos, with the goal of understanding their functions, their interactions, and their contribution to biological processes. Similarly, metabolomics has the goal to catalogue and quantify the entire range of metabolites. The emerging field of lipidomics focuses on the whole variety of lipid molecules.

The “omics revolution” is closely related to recent technical revolutions in mass spectrometry (MS). MS is a well established yet still a rapidly expanding technique in both industry and academia. It is widely and routinely used for biomolecule analysis aimed at drug discovery, diagnostics, and comprehensive assessment of biological, physiological, and pathological conditions [27].

Regarding embryo biotechnologies, MS-based omic applications are of special interest due to the high amount of structural molecular information data obtained from minimal amounts of samples. Introduction and development of omic studies can decisively contribute to the comprehension of yet unanswered questions regarding preimplantation embryo *in vitro* embryo metabolic needs, gamete cryopreservation and genetic reprogramming.

Besides possessing many centers for human assisted reproductive technologies (ART), most bovine *in vitro* fertilized embryos in the World are produced in Brazil due to a combination of several contributing factors such as herd size, the Zebu breed preponderance, and the high prices of these animals [48]. Commercial bovine cloning has also showed the highest efficiency in Brazil [47]. In this review, we summarize the basic MS concepts and instrumentation, and the current strategies used for proteomics, metabolomics and lipidomics MS studies with examples of recent applications in reproductive biotechnology.

II. MOLECULAR STRUCTURAL INFORMATION FROM YOUR SAMPLE: “CALL MASS SPECTROMETRY!”

MS is a unique analytical technique, which as seen incredible growth over the past 25 years, evolving to the forefront of analytical techniques. It is able to count and measure the mass of a great variety of isolated gaseous atoms and molecules in ionized forms in a fast, selective, highly sensitive and reliable way. MS differs therefore from other spectroscopic techniques such as ultraviolet, infrared, and nuclear magnetic resonance spectroscopies, which are based on the measurement of physical events resulting from the interaction of organic molecules with electromagnetic radiation. Currently, MS is a fundamental technique for characterization and quantitation of atoms

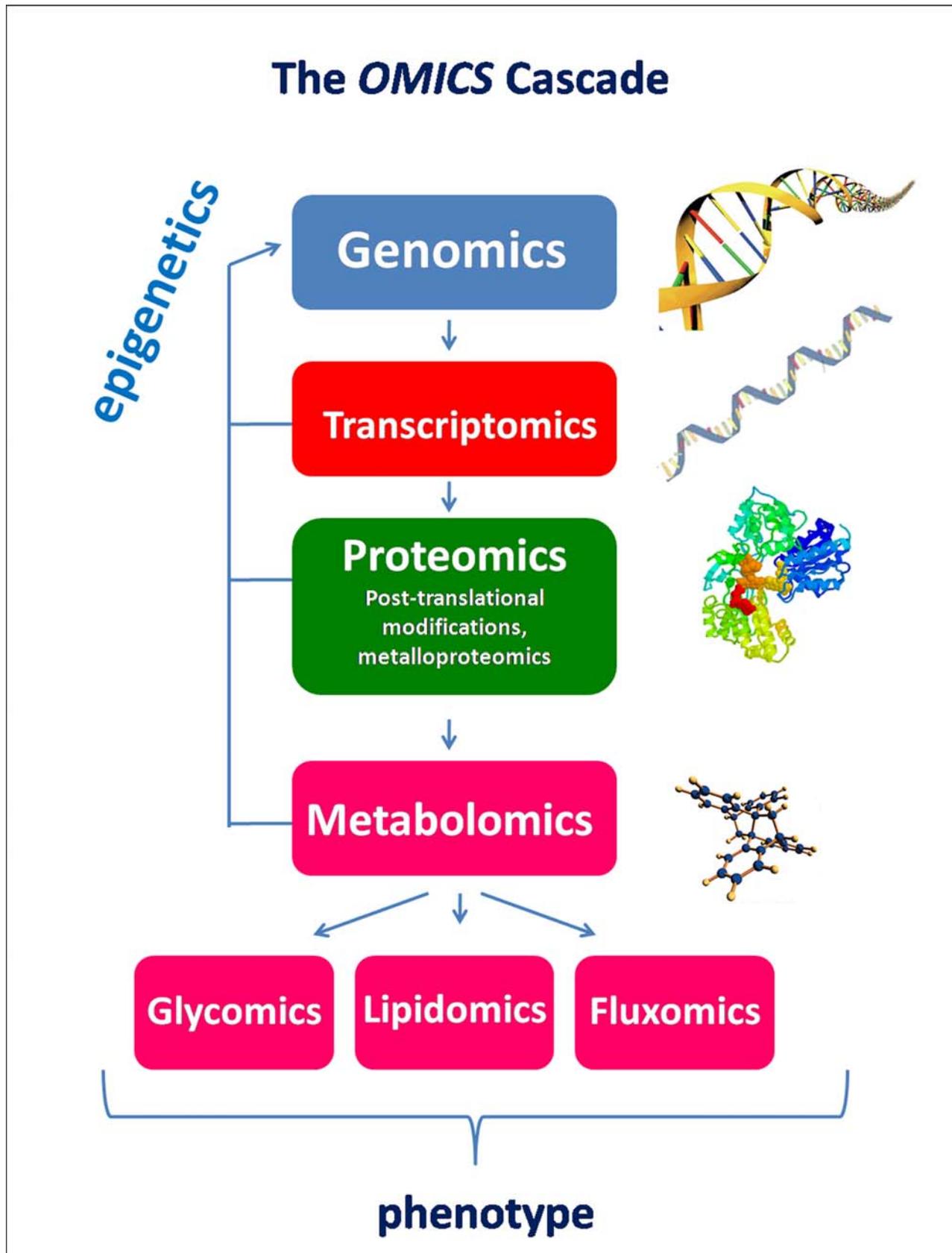


Figure 1. Schematic representation of the development of the omic fields. The “omics fever” began with the genome sequencing of whole organisms (genomics) and spread over to metabolomics, with the goal of characterizing the whole range of metabolites. Then again, gene expression can be altered by non-related DNA factors (epigenetics). The omic approach aims at integrating biological information to better understand the biological logic, complexity and information flux.

and molecules in chemistry, biology and medical sciences. MS instrumentation has increased approximately 5-fold in sensitivity every three years, allowing to study biomolecules in inconceivable ways a quarter of century ago [63].

Important applications of MS in biological sciences include the structural characterization of biomolecules such as carbohydrates, nucleic acids and steroids, sequencing of peptides and proteins and oligosaccharides, drug metabolism determination and quantification. MS can be used even to produce chemically selective images by monitoring the patterns of distribution of (bio)molecules in tissues via a technique known as MS imaging [15].

The atomic or molecular species analyzed by MS must be electrically charged and be in the gas phase to allow for manipulation inside the mass spectrometer, which weights each individual gaseous species by their mass-to-charge (m/z) ratios. These intact species can also be fragmented to access information of molecular connectivity (structure). MS allows therefore for the identification, quantification, and also elucidation of molecular structure.

Mass spectrometers are generally composed of three fundamental parts: a) the ionization source, b) the mass analyzer, and c) the detector. In the MS analysis flow (Figure 2), ions from the (bio)molecules of interest are generated via an appropriate ionization technique, separated according to the m/z values in a mass analyzer, and detected and counted (ion current) via an ion detector. The detector "counts" the ions by measuring the ion current and transforms such current into an electric pulse (a peak in the mass spectra). The data generated (mass spectrum) include the m/z of the ionic molecules or atoms on the abscissa and their relative abundance on the ordinate (peak heights) after normalization to the most abundant ion [55].

Initially, the application of MS was restricted to the analysis of gas, volatile, and thermally stable molecules due to limitations of the first ionization techniques such as electron ionization (EI) and chemical ionization (CI). In the 90's, however, two revolutionary techniques were developed for the ionization of large and labile biomolecules ionization. One of these was named electrospray ionization (ESI), which ionizes molecules and biomolecules in solutions and then "ejects" the solution ions into the gas phase. As elegantly summarized by the Nobel laureate John Fenn, "ESI makes molecular elephants fly". ESI has therefore allowed the coupling of MS to liquid chromatography: LC-MS [16]. The second revolutionary ionization technique was named matrix-assisted laser desorption ionization (MALDI; [37]). Both techniques had their importance recognized in the 2002 Chemistry Nobel Prize.

In summary, ESI-MS involves the spraying of the analyte solution through a thin metal capillary and the application of a strong electric field on the capillary tip. A very fine spray of highly charged (either positively or negatively charged) droplets will be formed and desolvated by a heated inert gas up to the point where the repulsive coulombic forces approach the magnitude of the surface tension forces [42] causing coulombic explosion and "ejection" of the analyte ions to the gas phase. Other ionization sources that can handle analytes in solution such as atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) can be also coupled to LC for omic studies, but they rely on the evaporation of neutral molecules from the spray droplets and post gas phase ionization.

For MALDI-MS, the sample can be only analyzed off-line and normally a small volume (1 or 2 μ L) of its solution is mixed with an organic matrix on a metallic target plate. Due to its pre-selected chemical nature, the matrix preferentially absorbs the laser beam energy and is desorbed rapidly carrying the analyte molecules to the gas phase. Within this high density plume of gaseous neutral and ionic species, a series of ion/molecule reactions promote analyte ionization. MALDI promotes selective heating of the matrix molecules and thus can handle heavy and non-volatile biomolecules such as proteins, polymers and oligonucleotides [26,44].

Modern mass analyzers include quadrupole mass-filters (Q), 3D ion-traps (IT), linear ion-traps (LIT), ion cyclotron resonance (ICR) traps, orbitraps, and time-of-flight (TOF) analyzers. Each analyzer displays a set of advantages and drawbacks. Some display ultra high resolution and accuracy for the m/z measurements. Nonetheless, due to their high cost and more demanding maintenance, other mass spectrometers may be more suitable because of being less expensive, robust, compact and easier to operate at the cost of low resolution and accuracy for the m/z measurements. Some mass spectrometers are perfect for quantitation whereas others display their best performance for high throughput analysis or the best cost/benefit ratio for *de novo* molecular characterization. Some of these analyzers can also be connected in sequential arrangements to allow for multi-stage MS, such as in triple (QqQ) or even pentaquadrupoles (QqQqQ) of TOF-TOF instruments [13]. Hybrid instruments are particularly clever arrangements since they try to combine two analyzers using each member of the pair to compensate the drawbacks of the other (MSⁿ capabilities of one analyzer with high resolution and accuracy for the other) hence hybrids provide overall the best MS performance. Most successful hybrids are the Q-traps (triple quadrupoles with a quadrupole that can be operated also as an LIT),

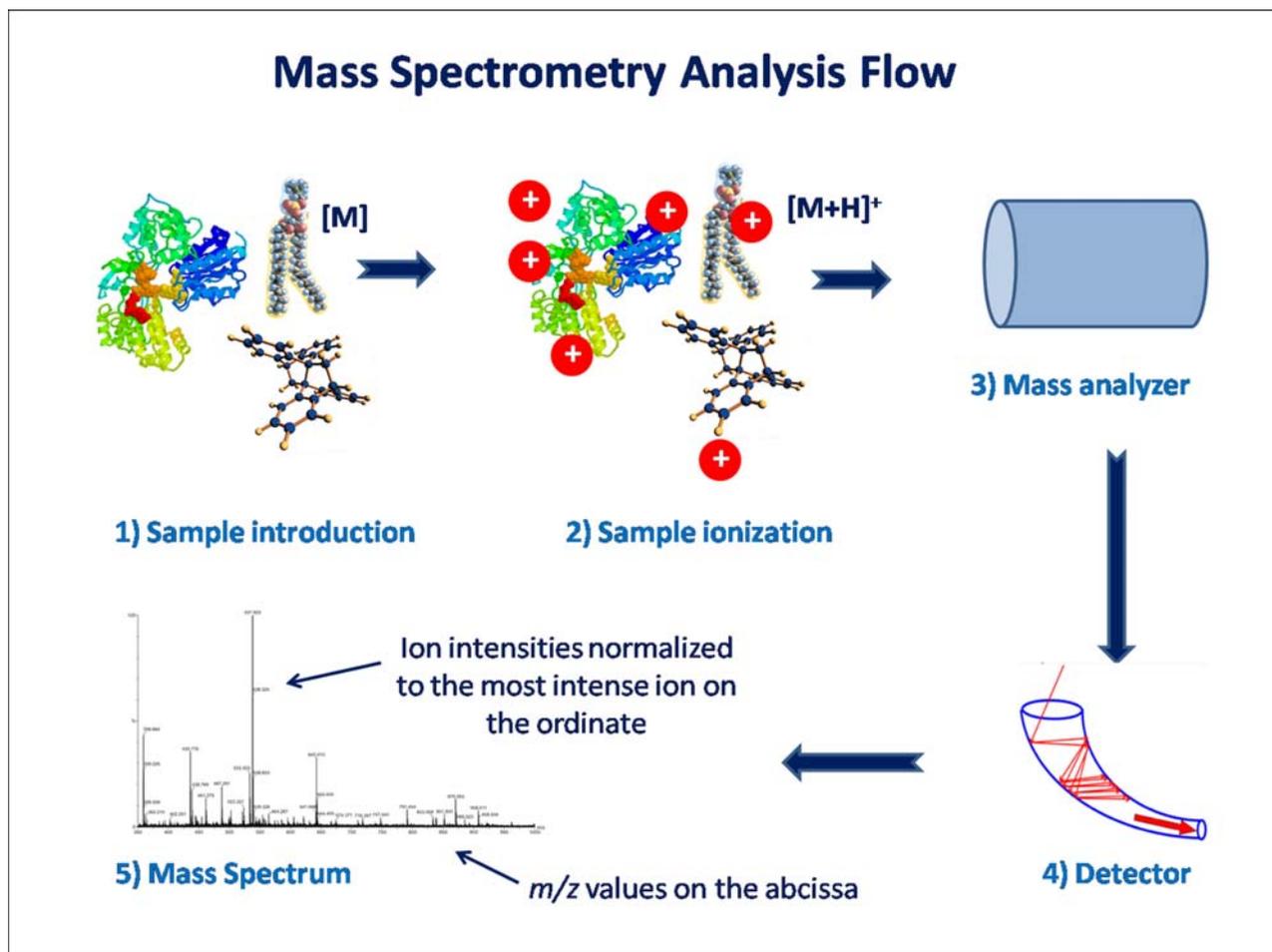


Figure 2. Schematic representation of the MS analysis flow, which begins by sample introduction and ionization. Molecules (M) or atoms need to be converted in positive ionic species by protonation (and therefore being represented as M^+ or $[M + H]^+$) or negative ionic species generated by deprotonation (and therefore being represented by $[M - H]^-$) for instance, are then conducted through a mass analyzer where they are separated according to m/z and then detected and counted. The m/z value and ion abundance are registered in the form of a peak in the mass spectrum.

IT-TOF, Q-TOF, Q-ICR and LIT-ICR, which also confers the advantage of structural analysis of biomolecules by dissociation via tandem (MS/MS) or multi-stage (MS^n) mass spectrometry experiments [27]. Mass spectrometers are operated in medium to very high vacuum (typically 10^{-3} to 10^{-11} Torr) to allow the sole ionization of analyte molecules and/or the undisturbed manipulation of the analyte ions. Mass spectrometric data are highly dependent on the sample introduction strategy, on the method of ionization and on the mass analyzer used. Therefore, instrumentation is an important issue for best performance according to each application [45].

Since a mass spectrometer separates, measures and detects ions of different masses, the different isotopes of a given element or different isotopologues of a given molecule are easily detected in the same abundances that they occur in nature, providing therefore typical isotopic “signatures” for atoms and molecules. For instance, organic molecules will display corresponding isotopologue ions with characteristic relative abundances due to ^{13}C (1.1% of natural abundance) as a function of the number of carbon atoms (11.0% for C_{10} , for instance) or monobrominated molecules will display a typical pattern of two peaks of about the same abundance separated by 2 m/z units (^{79}Br and ^{81}Br of ca the same natural abundance). Therefore, the mass spectrum provides typical isotopic patterns (isotopic signatures) that are very useful for analyte characterization and the identification of the presence of elements in the molecule composition, particularly for those elements with a rich and characteristic isotopic diversity, such as Cl, Br, S, Si, B and most of the metals [25]. Monoisotopic elements such as F and I also provide a clue since they produce no isotopic diversity.

Other relevant MS information is the overall mass defect of the molecule. The mass of a particular atom, except the ^{12}C of exactly 12 Da by convention, slightly deviates from integral masses, that is, is not exactly equal to

the sum of the masses of the individual neutrons, protons, and electrons of which the atom consists. This mass defect, due to different nucleus stabilities, can be measured if high resolution and accuracy mass spectrometers such as FT-ICRs are used. These instruments are capable of determining the “exact mass” of an ion at sub-ppm accuracy and hence to determine undoubtedly its full composition. Such data is also able to distinguish between isobars, which are molecules with the integral nominal mass (30 Da) but different exact masses such as C₂H₆ (30.04695), CH₂O (30.01056), and NO (29.99799). This feature is particularly useful for complex mixtures such as crude oil analysis [1]. Metabolomics strategies take great advantages of high resolution and accuracy MS to get molecular composition and to different small molecule isobars [25]. As an example, Figure 3 depicts the spectra in the positive and negative ion modes of lipids extracted from human follicular fluid (FF) obtained in few seconds by direct injection ESI of the lipid extract using an LTQ Fourier Transform-Ion Cyclotron Resonance (FT-ICR) mass spectrometer. The ultra-high resolution and accuracy for the *m/z* measurement and the possibility to fragment the ions allow structural analysis and confident attribution of the lipid species.

III. MS IN “THE QUEST” FOR PROTEIN BIOMARKERS

Biological processes are exquisitely orchestrated by dynamic signaling networks of interacting proteins. Even though gene expression studies are valuable, many reports have shown low levels of correlation when paired comparisons of mRNA changes and protein expression levels are performed [28,61]. This low correlation occurs because mRNA and protein degradation takes place during regulation of transcription and translation of nuclear DNA. The proteomics era is coincident with significant developments in MS, as well as fast bioinformatics tools, database search engines, and fulfillment of genome sequencing efforts. Proteomics involves not only the identification of gene products and their abundances, but also the use of protein interactomes for analysis of protein complexes, protein-protein interaction networks, and the dynamic behavior of the networks as a function of time or experimental condition. The major breakthrough of MS-based proteomics for biomarker search is the possibility to quantify a wide spectrum of proteins, and the easiness of assembling multiplex detection in a single measurement, an approach believed to lead to personalized medicine and treatments in the future [46].

MS ability to analyze proteins is the major driving force in the field of proteomics. Traditional MS-based proteomics strategies employ mostly MALDI-TOF MS or MALDI-TOF/TOF MS to obtain peptide mass fingerprints of protein digests of proteins individually isolated using 2-D gel electrophoresis. For instance, using 2D-gel electrophoresis followed by proteins isolation and digestion, a comprehensive profile of the abundant proteins of ovoid (at day 11 of gestation) and elongating (at day 12 of gestation) pig embryos was performed [10]. After MALDI-TOF MS and LC-MS/MS analysis of 305 isolated spots, 275 proteins were identified and 174 of them were found to be distinct. Differentially up-regulated proteins spanned a variety of functional categories, such as heat-shock proteins, translational factors, metabolic enzymes, cytoskeletal elements, and signal transducers, which helped to better comprehend biological mechanisms underlying the crucial stage of elongation in pig conceptuses.

MS-proteomics analysis in embryos has been recently reported for porcine [57], mouse [38] and human embryos [39], bringing new information regarding protein profile as a function of oxygen concentration during embryo *in vitro* culture and between different stages of development.

In assisted reproductive technologies (ART), the proteome is of particular interest in non-invasive gamete and embryo assessment by means of the secretome, e.g. those proteins produced by cells and secreted in the surrounding medium [33]. Unique proteins have been found in the human embryo secretome after activation of the embryonic genome, with special meaning for the ubiquitin, a component of the cell protein targeting for degradation and involved in proliferation and apoptosis [40]. Potential protein biomarkers in the secretome of human embryos have also been identified and are under validation [41].

MS-proteomics strategies known as the shotgun or bottom-up approaches are a valuable tool for high-complex and large-scale protein analysis. They are non-gel based strategies, thus avoiding the work-intensive, time-consuming and less efficient 2-D electrophoresis-based proteomics. Shotgun proteomics is equivalent to shotgun genomic sequencing, in which the DNA is fragmented, sequenced, and overlapped to “reconstruct” the whole sequence. Shotgun proteomics is based on LC-MS/MS, and is considered currently the leading proteomics technology. Yates *et al.* [62] also introduced the MudPIT (multidimensional protein identification technology) approach for 2-D LC-MS/MS

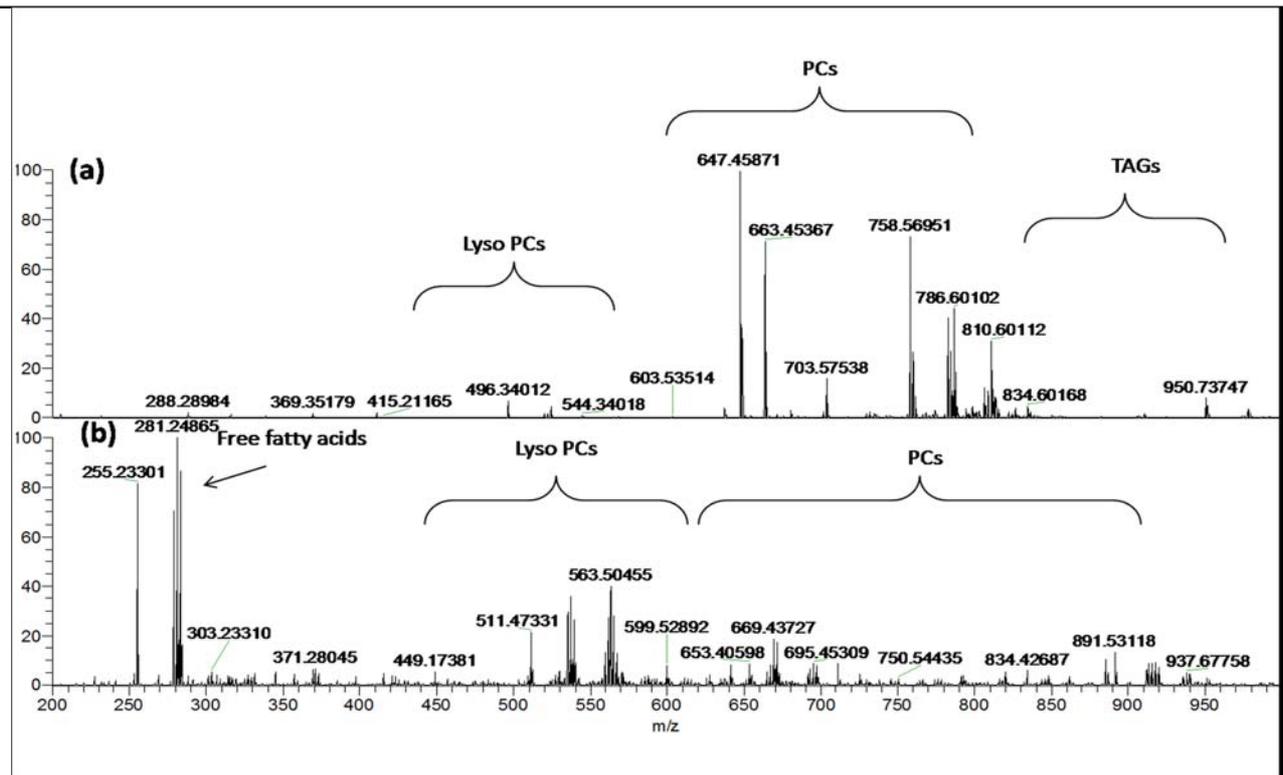


Figure 3. ESI FT-ICR mass spectra in the (a) positive and (b) negative ion modes of lipids extracted from human follicular fluid (FF). Data were obtained in few seconds by direct injection of the lipid extract into the ESI ion source of the LTQ FT-ICR mass spectrometer. PCs: phosphatidylcholines; TAGs: triacylglycerols.

proteomics. In MudPIT, the tryptic digests of all proteins contained in a biological sample (e.g. a cell lysate) are separated according to acidity in the first LC run and hydrophobicity in the second LC run. The 2D-LC eluted peptides are submitted then to ESI-MS/MS thus producing a set of fragment ions. The m/z values of these fragment ions correlated to their 2D-LC retention times allow the identification of individual proteins in the sample. MudPIT has been shown to identify a greater number of proteins as compared to traditional gel-based MS approaches [3, 11, 43, 49]

Follicular fluid (FF) is considered an interesting target for omic techniques in ART, especially proteomics, since it represents the natural microenvironment for oocytes during maturation and embryo development [20]. A bottom-up MS proteomics approach has identified 69 proteins in human FF, and among them, 32 not previously reported when using other proteomics approaches. Relevant proteins for follicle physiology such as sex hormone binding globulin and inhibin A were characterized [32].

Usually, proteomics data is used to compare different states of a specific organ or the whole organism, such as a healthy *versus* a diseased tissue. Quantitation is therefore a must in such comparative studies, and the power of MS-proteomics for quantitation has been increased dramatically by the use of isotope labeling [64]. Amino acids (AA) possessing light or heavy isotopes (as ^{13}C or ^{15}N) will behave rather similarly in biological systems and in sample preparation (tryptic digestion and LC), but the mass spectrometer, as discussed, is able to separate, measure and quantify each light and heavy isotopologues of the same molecule. Major isotope labeling MS-proteomics strategies are known as ICAT (isotope coded affinity tags), iTRAQ (isobaric tags for relative and absolute quantification), and SILAC (stable-isotope labeling by AA in cell culture) [24].

Stable-isotope strategies allow confident protein quantification, but “label-free” methods are also emerging as attractive alternatives. These strategies involve the redundant peptide-counting for estimating the abundance of a given protein in a LC-MS/MS run. MS-based proteomics can also be used for protein post-translational modifications (PTM) studies. PTM play pivotal roles in protein activity regulation, and PTM quantification provides a better understanding of disease mechanisms, cell differentiation and pluripotency, and facilitates the discovery of biomarkers for molecular classification of disease and cell differentiation stages [46].

IV. METABOLOME: THE “INFORMATION SOUP”

Cells produce a myriad of metabolites, which are the functional end products of all biological processes, and therefore a promising form of non-invasive methods for embryo selection in the ART field. Metabolomics analysis approaches are aimed at identifying and quantifying target intracellular and extracellular metabolites usually with MW lower than 1.000 Da, and have traditionally used techniques such as nuclear magnetic resonance (NMR) and fourier transform infrared spectroscopy (FTIR) [41]. Nonetheless, due to the unmatched combination of speed, sensitivity and selectivity, MS approaches are becoming the most important techniques in current metabolomics studies.

Compared to NMR, MS metabolomics presents higher sensitivity, higher-throughput and better identification power for unknowns in mixtures, with greater simplicity by eliminating or minimizing sample workup, pre-separation and chemical manipulation. For highly complex mixtures, coupling to LC may be required particularly for accurate quantitation, and hundreds or thousands of LC-MS/MS spectra with either intact or fragments of the molecular species recorded. Target analysis or metabolite profiling are the basic strategies of metabolomics. Targeted analysis focuses usually on specific pathways and involves quantitative analysis. Metabolite profiling is the scanning of a large number of intracellular metabolites under a defined condition [59]. Metabolite footprinting has been proposed as the measurement of all extracellular metabolites present in culture medium samples, which have been secreted by cells or organisms [2].

Metabolome data involve the variability at the atomic level present in small molecules, making it more complex than genomics (made up from four bases) and proteomics (made up from 22 aminoacids). To address this diversity, the metabolome can be subdivided into other omics such as the lipidomics, glycomics and peptidomics. The tremendous amount of information generated by metabolome analysis has been tackled with increasing efficacy by advances in bioinformatics. Compared to transcriptomics and proteomics, metabolomics is also considered a superior measure of cellular activity, especially for oocytes and embryos, since mRNA levels may fail to correlate with increased protein expression, and proteins may not be enzymatically active [28,54].

The use of metabolomics has been of especial interest for non-invasive assessment of oocyte and embryo quality [6,54]. Routinely, morphological assessment (e.g. cleavage timing, pronuclear formation, blastomeres fragmentation, blastocyst development, cell number) is the primary method used for evaluation of embryos from humans and domestic animals during *in vitro* culture, because of its speed, convenience and low cost. Nonetheless, morphologically normal embryos may not be viable or may harbor genetic and epigenetic defects [50,53].

Relevant information regarding oocyte and embryo viability has used targeted metabolomic approaches for amino acids analysis using conventional chromatography [4,5,7,34]. As an example, the turnover of three amino acids in human embryos culture medium (asparagine, glycine and leucine) was correlated with pregnancy and live birth, which in turn were not associated with maternal age and embryo cell number or morphological grading [7]. Metabolomic profiling of follicular fluid or culture media has the potential to become a high throughput non-invasive platform to assess gamete and embryo viability, bringing significant contributions to human ART and in animal embryo biotechnologies [54]. A simple approach involving MS fingerprinting profile has been recently demonstrated as a fast and high-throughput strategy to control the quality of bovine embryo culture medium [19].

V. MAKING LIPIDS FLY BY MS

Driven by the pivotal role of lipids in cell membrane structure and in cell signaling and metabolism, the field of metabolomics has extended into lipidomics. As observed in other omic technologies, the lipidomic field has also been driven by the recent “MS revolution”. The lipidome from mammalian organisms are represented by a great variety of tens of thousands lipid species [58]. For instance, different fatty acids (FA) residues can be attached to the glycerol backbone of monoacylglycerols (MAGs), and most dramatically in different combinations and positions (stereochemistry) for diacylglycerols (DAGs) and triacylglycerols (TAGs). Also, the glycerol backbone can contain not only FAs, but also polar head groups forming the monoacylglycerolphosphates (lysophospholipids), and diacylglycerolphosphates (phospholipids). FAs also display their diversity in terms of carbon number and unsaturation and the *sn*-1 position in phospholipids can be substituted for ether or vinyl ether linkages, forming the plasmalogen and plasmalogen glycerolphospholipids [31,45].

For decades, thin layer chromatography (TLC) and gas chromatography (GC) were the methods of choice for the separation of lipid into their classes. The protocol for lipid analysis by GC is time-consuming and involves hydrolysis and derivatization of relatively large amounts of lipids, and identification of individual FAs requires MS coupling via GC-MS. Using ESI and MALDI, lipid analysis has been revolutionized by MS allowing intact lipids to be detected rapidly and directly from their natural matrices with no or little sample workup and no chemical manipulation. After detection of single molecular species such as M^- or M^+ for the anionic or cationic lipids and $[M + H]^+$ or $[M - H]^-$ for the neutral lipids, collision-induced dissociation via MS/MS experiments can be used to unambiguously characterize each lipid in such molecular ionic species. Five major classes are best detected in the positive ion mode: phosphatidylcholines (PC), phosphatidylserines (PS), phosphatidylethanolamines (PE), sphingomyelins (SM), and triacylglycerols (TAGs), and six in the negative ion mode: phosphatidylinositols (PI), phosphatidylserines (PS), phosphatidylglycerols (PG), glycerophosphatidic acids (PA), PE, and chloride adducts of PCs [45].

Numerous studies of cellular lipidome have demonstrated that lipid analysis by ESI-MS(/MS) represents an unparalleled approach for lipidomics due to its unmatched sensitivity (range of fmol to pmol of lipid/ μ L), selectivity (discriminating power) and structural assignments. ESI-MS(/MS) allows quantitation of lipid classes, subclasses, or even individual species in minutes by direct injection of a lipid extract, or after LC separation, demonstrating a nearly linear relation between the relative intensities and concentration, with excellent reproducibility (>95%) [29-31,35,36]

State-of-the-art web-based tools of data analysis are well represented by the LIPID MAPS initiative, which together with the International Committee for the Classification and Nomenclature of Lipids (ICCNL) has organized the lipid nomenclature and classification. Tools for analysis of lipids are also available in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, as well as various software tools and databases which can be used for lipidomics data processing and pathway analysis [60].

Two lipidomic protocols are mainly used. The first involves the use of selective extraction optimized for each lipid category, followed by their LC separation. The second is known as “shotgun lipidomics”, and involves the direct MS analysis of lipid extracts without previous separation by selective extraction. ESI, MALDI MS or MS/MS experiments and the use of internal standards provide lipid characterization and quantification [12].

A recent shotgun lipidomic MS study was successful at characterizing and describing the detailed metabolic pathway of 250 lipids (corresponding to 21 lipid classes) from *Saccharomyces cerevisiae* membranes, which corresponded to ~95% of the lipid variety present in the yeast. Changes in entire lipidome profiles were observed when culture temperature varied, or after genomic deletion of 3-ketoacyl-CoA synthases [14].

Since lipids are involved in membrane dynamics, cell energy homeostasis and regulation of the molecular machinery, they may serve as an excellent source of information when accelerated cell growth occurs in cancer cells. Several lipid classes such as glycerophospholipids, sphingolipids, lysolipids, and oxidized fatty acids have been reported to be altered in cancer conditions. Due to the major advantages regarding sensitivity, accuracy, reproducibility and applicability in detection and quantitation of a large array of lipid molecules, LC-MS(/MS) is being employed in the search of cancer biomarkers based on individual lipids, a class of lipids, or a unique lipid fingerprint [17].

ESI is considered the ionization technique of choice for MS lipidomic studies, but MALDI is as useful for lipid fingerprinting of biological samples [21-23,51,52]. We have used MALDI-MS to obtain direct lipid fingerprinting of individual oocytes and embryos of various species [58]. Due to the sensitivity and easiness of lipid ionization by MALDI, no sample extraction was needed. Embryos and oocytes were simply placed on a target plate, and covered with the MALDI matrix. PCs, SMs and TAGs were detected in few seconds. Oocytes and embryos displayed different lipid profiles, and embryos cultured in low oxygen atmosphere (5%) and without fetal calf serum as a supplement, showed PCs with less palmitic acid (a saturated fatty acyl residue) and more oleic acid (an unsaturated fatty acyl residue) [18]. Owing to MALDI-MS imaging technology, precise spatial and temporal differences in phospholipid composition during embryo implantation have also been revealed [8,9]

VI. “REAL-WORLD” PERSPECTIVES OF OMIC APPLICATIONS ON EMBRYO TECHNOLOGIES

To our knowledge, a number of projects, experiments and new applications involving MS-based omic strategies specifically designed for embryo and gamete biotechnology area are underway or have their suitability already reported.

The use of MS fingerprinting for controlling the quality and evaluating the stability of culture media used for bovine embryo *in vitro* production [19] has contributed with improving the routine for producing and storing commercial culture media. In these studies, MS analysis enabled the identification, of subtle changes in the chemical profile of embryo and oocyte culture media caused by temperature fluctuations, further demonstrating how omics can be used to establish new strategies of media storage and delivering.

MALDI-MS lipid fingerprinting of individual oocytes and embryos is a fast and high-throughput technique [18], which can add valuable information to experiments aimed at optimizing embryo *in vitro* culture systems for cryopreservation. Also the detection of the "ideal" lipid profiles obtained from *in vivo*-derived embryos can be used as an indicative of embryo cryosensitivity. For semen analysis, MALDI-MS lipid fingerprinting [21,23] can become a tool for the early evaluation of bull semen, especially if lipid biomarkers related to sperm cryoresistance or cryosensitivity can be characterized.

Also, the characterization of the swine and bovine follicular fluid environment regarding the protein, amino acids and lipid content can contribute with the development of new synthetic media for embryo *in vitro* production allowing higher embryo development and viability, with increase in pregnancy rates and cryopreservation success.

Another interesting perspective is related to the need of increasing commercial success of animal cloning. The study of histone PTM can help to understand the control of cellular gene expression and repression patterns, which have been extensively associated to lower success rates in animal cloning [56].

VII. CONCLUSIONS

Modern and powerful MS methods are revolutionizing the fields of proteomics, lipidomics and metabolomics. This MS revolution has encouraged researches to value the individual characterization and quantitation of biomolecules in living systems, as well as the need of a systemic molecular biological view. As an illustrative example, for embryo *in vitro* production studies, MS can bring key contributions by monitoring the highly dynamic metabolic changes during cell replication and differentiation, response to temperature, or oxidative stress.

Hand portable mass spectrometers are also being now commercialized decreasing the cost and operation requirements of MS machines, which are in unprecedented manner facilitating the spread and routine use of MS in research and commercial settings. The power and universality of MS in the whole biological sciences is now becoming to be fully realized. In Brazil, where MS competence is internationally recognized, human resources training and the establishment of multi user MS centers are the main challenges for the successful introduction of omics sciences in the reproductive biotechnology field.

Acknowledgements. We thank FAPESP (grant 2008/10756-7) and CNPq for financial support.

REFERENCES

- 1 Alberici R.M., Simas R.C., de Souza V., de Sa G.F., Daroda R.J. & Eberlin M.N. 2010. Analysis of fuels via easy ambient sonic-spray ionization mass spectrometry. *Analytica Chimica Acta.* 659(1-2): 15-22.
- 2 Allen J., Davey H.M., Broadhurst D., Heald J.K., Rowland J.J., Oliver S.G. & Kell D.B. 2003. High-throughput classification of yeast mutants for functional genomics using metabolic footprinting. *Nature Biotechnology.* 21(6): 692-696.
- 3 Bassnett S., Wilmarth P.A. & David L.L. 2009. The membrane proteome of the mouse lens fiber cell. *Molecular Vision.* 15: 2448-2463.
- 4 Booth P.J., Humpherson P.G., Watson T.J. & Leese H.J. 2005. Amino acid depletion and appearance during porcine preimplantation embryo development in vitro. *Reproduction.* 130(5): 655-668.
- 5 Booth P.J., Watson T.J. & Leese H.J. 2007. Prediction of porcine blastocyst formation using morphological, kinetic, and amino acid depletion and appearance criteria determined during the early cleavage of in vitro-produced embryos. *Biology of Reproduction.* 77(5): 765-779.

- 6 Botros L., Sakkas D. & Seli E. 2008. Metabolomics and its application for non-invasive embryo assessment in IVF. *Molecular Human Reproduction.* 14(12): 679-690.
- 7 Brison D.R., Houghton F.D., Falconer D., Roberts S.A., Hawkhead J., Humpherson P.G., Lieberman B.A. & Leese H.J. 2004. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Human Reproduction.* 19(10): 2319-2324.
- 8 Burnum K.E., Tranguch S., Mi D., Daikoku T., Dey S.K. & Caprioli R.M. 2008. Imaging mass spectrometry reveals unique protein profiles during embryo implantation. *Endocrinology.* 149(7): 3274-3278.
- 9 Burnum K.E., Cornett D.S., Puolitaival S.M., Milne S.B., Myers D.S., Tranguch S., Brown H.A., Dey S.K. & Caprioli R.M. 2009. Spatial and temporal alterations of phospholipids determined by mass spectrometry during mouse embryo implantation. *Journal of Lipid Research.* 50(11): 2290-2298.
- 10 Degrelle S.A., Blomberg L.A., Garrett W.M., Li R.W. & Talbot N.C. 2009. Comparative proteomic and regulatory network analyses of the elongating pig conceptus. *Proteomics.* 9(10): 2678-2694.
- 11 Delahunty C.M. & Yates J.R. 2007. MudPIT: multidimensional protein identification technology. *Biotechniques.* 43(5): 563-569.
- 12 Dennis E.A. 2009. Lipidomics joins the omics evolution. *Proceedings of the National Academy of Sciences U S A.* 106(7): 2089-2090.
- 13 Eberlin M.N. 2007. Electrospray ionization mass spectrometry: a major tool to investigate reaction mechanisms in both solution and the gas phase. *European Journal of Mass Spectrometry.* 13(1): 19-28.
- 14 Ejsing C.S., Sampaio J.L., Surendranath V., Duchoslav E., Ekroos K., Klemm R.W., Simons K. & Shevchenko A. 2009. Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. *Proceedings of the National Academy of Sciences U S A.* 106(7): 2136-2141.
- 15 Feng X., Liu X., Luo Q. & Liu B.F. 2008. Mass spectrometry in systems biology: an overview. *Mass Spectrometry Reviews.* 27(6): 635-660.
- 16 Fenn J.B., Mann M., Meng C.K., Wong S.F. & Whitehouse C.M. 1989. Electrospray Ionization for Mass-Spectrometry of Large Biomolecules. *Science.* 246(4926): 64-71.
- 17 Fernandis A.Z. & Wenk M.R. 2009. Lipid-based biomarkers for cancer. *Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences.* 877(26): 2830-2835.
- 18 Ferreira C.R., Saraiva S.A., Catharino R.R., Garcia J.S., Gozzo F.C., Sanvido G.B., Santos L.F., Lo Turco E.G., Pontes J.H., Basso A.C., Bertolla R.P., Sartori R., Guardieiro M.M., Perecin F., Meirelles F.V., Sangalli J.R. & Eberlin M.N. 2010. Single embryo and oocyte lipid fingerprinting by mass spectrometry. *J Lipid Res.* DOI 10.1194/jlr.D001768. [in press].
- 19 Ferreira C.R., Souza G.H., Riccio M.F., Catharino R.R., Pontes J.H., Basso A.C., Junior J.C., Perecin F. & Eberlin M.N. 2009. Mass spectrometry fingerprinting of media used for in vitro production of bovine embryos. *Rapid Communications in Mass Spectrometry.* 23(9): 1313-1320.
- 20 Fortune J.E. 1994. Ovarian follicular growth and development in mammals. *Biology of Reproduction.* 50(2): 225-232.
- 21 Fuchs B., Muller K., Goritz F., Blottner S. & Schiller J. 2007. Characteristic oxidation products of choline plasmalogens are detectable in cattle and roe deer spermatozoa by MALDI-TOF mass spectrometry. *Lipids.* 42(11): 991-998.
- 22 Fuchs B. & Schiller J. 2008. MALDI-TOF MS analysis of lipids from cells, tissues and body fluids. *Subcellular Biochemistry.* 49: 541-565.
- 23 Fuchs B., Jakop U., Goritz F., Hermes R., Hildebrandt T., Schiller J. & Muller K. 2009. MALDI-TOF "fingerprint" phospholipid mass spectra allow the differentiation between ruminantia and feloidea spermatozoa. *Theriogenology.* 71(4): 568-575.
- 24 Gingras A.C., Gstaiger M., Raught B. & Aebersold R. 2007. Analysis of protein complexes using mass spectrometry. *Nature Reviews Molecular Cell Biology.* 8(8): 645-654.
- 25 Glish G.L. & Vachet R.W. 2003. The basics of mass spectrometry in the twenty-first century. *Nature Reviews Drug Discovery.* 2(2): 140-150.
- 26 Gluckmann M., Pfenninger A., Kruger R., Thierolf M., Karas M., Horneffer V., Hillenkamp F. & Strupat K. 2001. Mechanisms in MALDI analysis: surface interaction or incorporation of analytes? *International Journal of Mass Spectrometry.* 210(1-3): 121-132.
- 27 Griffiths W.J. & Wang Y. 2009. Mass spectrometry: from proteomics to metabolomics and lipidomics. *Chemical Society Reviews.* 38(7): 1882-1896.
- 28 Gygi S.P., Rochon Y., Franza B.R. & Aebersold R. 1999. Correlation between protein and mRNA abundance in yeast. *Molecular and Cellular Biology.* 19(3): 1720-1730.
- 29 Han X. & Gross R.W. 2001. Quantitative analysis and molecular species fingerprinting of triacylglyceride molecular species directly from lipid extracts of biological samples by electrospray ionization tandem mass spectrometry. *Analytical Biochemistry.* 295(1): 88-100.
- 30 Han X. 2002. Characterization and direct quantitation of ceramide molecular species from lipid extracts of biological samples by electrospray ionization tandem mass spectrometry. *Analytical Biochemistry.* 302(2): 199-212.
- 31 Han X. & Gross R.W. 2003. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics. *Journal of Lipid Research.* 44(6): 1071-1079.
- 32 Hanrieder J., Nyakas A., Naessen T. & Bergquist J. 2008. Proteomic analysis of human follicular fluid using an alternative bottom-up approach. *Journal of Proteome Research.* 7(1): 443-449.
- 33 Hathout Y. 2007. Approaches to the study of the cell secretome. *Expert Review of Proteomics.* 4(2): 239-248.
- 34 Houghton F.D., Hawkhead J.A., Humpherson P.G., Hogg J.E., Balen A.H., Rutherford A.J. & Leese H.J. 2002. Non-invasive

- amino acid turnover predicts human embryo developmental capacity. *Human Reproduction.* 17(4): 999-1005.
- 35 Ivanova P.T., Cerda B.A., Horn D.M., Cohen J.S., McLafferty F.W. & Brown H.A. 2001. Electrospray ionization mass spectrometry analysis of changes in phospholipids in RBL-2H3 mastocytoma cells during degranulation. *Proceedings of the National Academy of Sciences U S A.* 98(13): 7152-7157.
- 36 Ivanova P.T., Milne S.B., Byrne M.O., Xiang Y. & Brown H.A. 2007. Glycerophospholipid identification and quantitation by electrospray ionization mass spectrometry. *Methods in Enzymology.* 432: 21-57.
- 37 Karas M. & Hillenkamp F. 1988. Laser Desorption Ionization of Proteins with Molecular Masses Exceeding 10000 Daltons. *Analytical Chemistry.* 60(20): 2299-2301.
- 38 Katz-Jaffe M.G., Linck D.W., Schoolcraft W.B. & Gardner D.K. 2005. A proteomic analysis of mammalian preimplantation embryonic development. *Reproduction.* 130(6): 899-905.
- 39 Katz-Jaffe M.G., Gardner D.K. & Schoolcraft W.B. 2006. Proteomic analysis of individual human embryos to identify novel biomarkers of development and viability. *Fertility and Sterility.* 85(1): 101-107.
- 40 Katz-Jaffe M.G., Schoolcraft W.B. & Gardner D.K. 2006. Analysis of protein expression (secretome) by human and mouse preimplantation embryos. *Fertility and Sterility.* 86(3): 678-685.
- 41 Katz-Jaffe M.G., McReynolds S., Gardner D.K. & Schoolcraft W.B. 2009. The role of proteomics in defining the human embryonic secretome. *Molecular Human Reproduction.* 15(5): 271-277.
- 42 Kebarle P. & Tang L. 1993. From Ions in Solution to Ions in the Gas-Phase - the Mechanism of Electrospray Mass-Spectrometry. *Analytical Chemistry.* 65(22): A972-A986.
- 43 Kline K.G. & Wu C.C. 2009. MudPIT analysis: application to human heart tissue. *Methods in Molecular Biology.* 528: 281-293.
- 44 Knochenmuss R. 2006. Ion formation mechanisms in UV-MALDI. *Analyst.* 131(9): 966-986.
- 45 Milne S., Ivanova P., Forrester J. & Alex Brown H. 2006. Lipidomics: an analysis of cellular lipids by ESI-MS. *Methods.* 39(2): 92-103.
- 46 Pan S., Aebersold R., Chen R., Rush J., Goodlett D.R., McIntosh M.W., Zhang J. & Brentnall T.A. 2009. Mass spectrometry based targeted protein quantification: methods and applications. *Journal of Proteome Research.* 8(2): 787-797.
- 47 Panarace M., Aguero J.I., Garrote M., Jauregui G., Segovia A., Cane L., Gutierrez J., Marfil M., Rigali F., Pugliese M., Young S., Lagioia J., Garnil C., Pontes J.E.F., Junio J.C.E., Mower S. & Medina M. 2007. How healthy are clones and their progeny: 5 years of field experience. *Theriogenology.* 67(1): 142-151.
- 48 Pontes J.H., Nonato-Junior I., Sanches B.V., Ereno-Junior J.C., Uvo S., Barreiros T.R., Oliveira J.A., Hasler J.F. & Seneda M.M. 2009. Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (*Bos indicus*) donor cows. *Theriogenology.* 71(4): 690-697.
- 49 Richardson M.R., Price M.O., Price F.W., Pardo J.C., Grandin J.C., You J., Wang M. & Yoder M.C. 2009. Proteomic analysis of human aqueous humor using multidimensional protein identification technology. *Molecular Vision.* 152: 740-2750.
- 50 Sakkas D. & Gardner D.K. 2005. Noninvasive methods to assess embryo quality. *Current Opinion in Obstetrics and Gynecology.* 17(3): 283-288.
- 51 Schiller J., Suss R., Arnhold J., Fuchs B., Lessig J., Muller M., Petkovic M., Spalteholz H., Zschornig O. & Arnold K. 2004. Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry in lipid and phospholipid research. *Progress in Lipid Research.* 43(5): 449-488.
- 52 Schiller J., Suss R., Fuchs B., Muller M., Zschornig O. & Arnold K. 2007. MALDI-TOF MS in lipidomics. *Frontiers in Bioscience.* 12: 2568-2579.
- 53 Sinclair K.D., Lea R.G., Rees W.D. & Young L.E. 2007. The developmental origins of health and disease: current theories and epigenetic mechanisms. *Society for Reproduction and Fertility Supplement.* 64:425-443.
- 54 Singh R. & Sinclair K.D. 2007. Metabolomics: approaches to assessing oocyte and embryo quality. *Theriogenology.* 68 Suppl: 1S56-62.
- 55 Siuzdak G. 1994. The emergence of mass spectrometry in biochemical research. *Proceedings of the National Academy of Sciences U S A.* 91(24): 11290-11297.
- 56 Smith L.C. & Yoo J.G. 2009. Animal cloning by somatic cell nuclear transfer. *Methods in Molecular Biology.* 550: 267-279.
- 57 Sutovsky P., Manandhar G., Laurincik J., Letko J., Caamano J.N., Day B.N., Lai L., Prather R.S., Sharpe-Timms K.L., Zimmer R. & Sutovsky M. 2005. Expression and proteasomal degradation of the major vault protein (MVP) in mammalian oocytes and zygotes. *Reproduction.* 129(3): 269-282.
- 58 van Meer G. 2005. Cellular lipidomics. *Embo Journal.* 24(18): 3159-3165.
- 59 Villas-Boas S.G., Mas S., Akesson M., Smedsgaard J. & Nielsen J. 2005. Mass spectrometry in metabolome analysis. *Mass Spectrometry Reviews.* 24(5): 613-646.
- 60 Wheelock C.E., Goto S., Yetukuri L., D'Alexandri F.L., Klukas C., Schreiber F. & Oresic M. 2009. Bioinformatics strategies for the analysis of lipids. *Methods in Molecular Biology.* 580: 339-368.
- 61 Williamson A.J., Smith D.L., Blinco D., Unwin R.D., Pearson S., Wilson C., Miller C., Lancashire L., Lacaud G., Kouskoff V. & Whetton A.D. 2008. Quantitative proteomics analysis demonstrates post-transcriptional regulation of embryonic stem cell differentiation to hematopoiesis. *Molecular & Cellular Proteomics.* 7(3): 459-472.
- 62 Wolters D.A., Washburn M.P. & Yates J.R. 2001. An automated multidimensional protein identification technology for shotgun proteomics. *Analytical Chemistry.* 73(23): 5683-5690.

63 Zhou M. & Veenstra T. 2008. Mass spectrometry: m/z 1983-2008. *Biotechniques*. 44(5): 667-668, 670.

64 Zhu W., Smith J.W. & Huang C.M. 2010. Mass spectrometry-based label-free quantitative proteomics. *Journal of Biomedicine and Biotechnology*. Volume 2010, Article 840518, 6p.

