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REVIEW

Protein pathways working in human follicular fluid: the future for tailored IVF?

LAURA BIANCHI[1], ASSUNTA GAGLIARDI[1], CLAUDIA LANDI[1], RICCARDO FOCARELLI[2], VINCENZO DE LEO[3], ALICE LUDDI[3], LUCA BINI[1], PAOLA PIOMBONI[3]*

[1]*Functional Proteomics Laboratory, Department of Life Sciences, Siena University, Via Aldo Moro 2, 53100 Siena, Italy,* [2]*Department of Life Sciences, Siena University, Via Aldo Moro, 53100 Siena, Italy, and* [3]*Department of Molecular and Developmental Medicine, University of Siena, Viale Bracci 14, 53100 Siena, Italy*

The human follicular fluid (HFF) contains molecules and proteins that may affect follicle growth, oocyte maturation and competence acquiring. Despite the numerous studies, an integrated broad overview on biomolecular and patho/physiological processes that are proved or supposed to take place in HFF during folliculogenesis and oocyte development is still missing. In this review we report, for the first time, all the proteins unambiguously detected in HFF and, applying DAVID (Database for Annotation, Visualization and Integrated Discovery) and MetaCore bioinformatic resources, we shed new lights on their functional correlation, delineating protein patterns and pathways with reasonable potentialities for oocyte quality estimation in in vitro fertilisation (IVF) programs. Performing a rigorous PubMed search, we redacted a list of 617 unique proteins unambiguously-annotated as HFF components. Their functional processing suggested the occurrence in HFF of a tight and highly dynamic functional-network, which is balanced by specific effectors, primarily involved in extracellular matrix degradation and remodelling, inflammation and coagulation. Metalloproteinases, thrombin and vitamin-D-receptor/retinoid-X-receptor-alpha resulted as the main key factors in the nets and their differential activity may be indicative of ovarian health and oocyte quality. Despite future accurate clinical investigations are absolutely needed, the present analysis may provide a starting point for more accurate oocyte quality estimation and for defining personalised therapies in reproductive medicine.

Introduction

Assisted reproductive technologies (ART) have been recently more widely applied in order to answer the increasing needs of infertile couples asking for a pregnancy. Intracytoplasmic sperm injection (ICSI) is the most used technology accounting for around two-thirds of all treatments worldwide, while conventional in vitro fertilisation (IVF) is around one-third, with great variations of this proportion between Countries. Even if their effectiveness has improved in the past few years, outcome rates of each technique are comparable and the chance of pregnancy still remains roughly 35.5% per embryo transfer, with 4.5% live birth rate per mature oocyte retrieved (Ref. 1). Nevertheless, around 1.5 million ART cycles are performed each year worldwide, with an estimated 5 million births from assisted conceptions since the first IVF baby was born in 1978 (ESHRE, 2010; http://www.eshre.eu/Guidelines-and-Legal/ART-fact-sheet.aspx).

Taking into account this increase in ART application and the unsatisfactory percentage of positive results, the next challenge in reproductive biology is to both define biological factors and improve clinical procedures that may contribute to the positive outcome in assisted reproduction.

Even if endometrial receptivity plays a pivotal role in embryo implantation, many clinical data suggest that a great percentage of embryo implantation failure may be because of low embryo quality. The early embryo development and the ongoing pregnancy depend on oocyte competence, which is acquired during folliculogenesis. In this respect, mechanisms involved in oocyte development and competence acquisition may represent one of the main aspects to be investigated more in depth in order to reduce the high failure rate of ART (Ref. 2).

In the last years, several works have been performed to determine the protein composition of the human follicular fluid. The majority of such researches were mainly limited to the identification and description of the fluid components, without providing significant insights into the roles that these proteins may exert in follicle physiology and in IVF outcome (Refs 3, 4, 5, 6, 7, 8). Hence, a more deep comprehension of molecular processes would help clinicians to increase success in ART procedures also by treating specific categories of patients with a personalised approach.

The oocyte resides in a highly metabolically active micro-environment whose molecular dynamics are largely depicted by human follicular fluid (HFF)

biochemical properties. Follicular fluid originates by diffusion of blood proteins through the thecal capillaries and by secretions from granulosa and theca cells, and from oocyte, thus providing a special microenvironment, which is essential for the maturation of the oocyte. Follicular fluid is characterised by a huge protein complexity and a very wide dynamic range of proteins involved in numerous different pathways. Reflecting granulosa and theca metabolic status, HFF biochemical composition may reveal not only the functional state of the follicle itself, but also oocyte competence, which influences oocyte quality, fertilisation and embryo development. After oocyte recovery for IVF procedures, HFF is an abundant biological sample that can be easily collected without compromising the gametes quality. As a consequence, it represents an attractive target for the development of noninvasive assays for oocyte competence evaluation since, at present, morphological criteria are the main strategy for oocyte and embryo selection. Indeed, the molecular characterisation of this body fluid could lead to the discovery of biomarkers with diagnostic and predictive values for a wide range of fertility problems.

An increasing number of data from proteomic studies analysing the HFF protein profile is present in literature, but a functional correlation of all the identified proteins, obtained by different experimental investigations, was never attempted.

In this review, we present results obtained by a powerful strategy of data revision in which all the HFF proteins reported in relevant literature are processed at once applying bioinformatics tools, as DAVID (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov) and MetaCore (Thomson Reuters) resources, for functional clustering and pathway analysis, respectively.

Indeed, we provide a novel, wide, critical, and comprehensive functional-overview of the follicle milieu outlining the complex and integrated protein-framework in which the oocyte develops and acquires competence.

Follicle functional microenvironment: a high throughput in-silico approach

In the last years, several works have been performed to determine the protein composition of the HFF (Refs 3, 4, 5, 6, 7, 8, 9, 10, 11, 12) and the application of high sensitive mass spectrometry approaches has bona fide lead to the identification of the overwhelming majority of the fluid proteins (Refs 5, 13, 14, 15). However, several of those researches were mainly limited to the identification and description of the fluid components without their functional integration. Only in some cases, Authors suggested and discussed HFF supposed bioactivities, according to individual protein functions, and they provide significant insights into the roles that HFF-detected proteins may exert in follicle physiology and in IVF outcome (Refs 9, 16). In this regard, any clear correlation has not yet been described and/or proved, by large-scale clinical investigations, between altered HFF protein composition and reproductive performance. To our knowledge, no protein-biomarker of oocyte 'quality' has yet been identified and/or properly tested in the HFF and no extensive functional integration of all HFF protein constituents has ever been attempted before.

In this review, taking advantage of the DAVID (http://david.abcc.ncifcrf.gov) bioinformatic resource for functional clusterisation and of the MetaCore pathway analysis tool (Thomson Reuters), we provide a novel and critical functional overview on pre-ovulatory follicle activities delineating a complex and integrated functional protein-framework in which the oocyte has developed, differentiated and maturated. To this end, we performed a systematic PubMed-search for English language scientific papers, published between January 2000 and December 2014, using the combination of human AND 'follicular fluid' searching words. We then restricted our investigation to those papers that experimentally described HFF protein components. We included in the study only proteins whose presence was proved at protein level in HFF and that are nonambiguous factors corresponding to single specific amino acid sequences reported in UniProtKB (UniProt KnowledgeBase), and/or NCBI Protein (National Center for Biotechnology Information), and/or GenBank (Protein) databases. Among them, exclusively those proteins with a molecular weight >8 kDa were considered. Approximately 617 unique proteins from HFF satisfy stated parameters. All of them are listed in Supplementary Table I and the corresponding articles are reported in Supplementary References. Methodological details about PubMed search, protein selection, table redaction and Bioinformatics analyses are specified in Supplementary Methods.

Biological-function clustering of HFF proteins

Among the 617 factors that the extensive literature search designated as HFF unique and nonambiguous proteins, 337 protein factors (Supplementary Table II) were clustered into five, statistically significant ($P < 0.001$), main groups by the DAVID enrichment analysis tool according to their GO biological functions (Supplementary Methods and Table II). Protein distribution into these groups was visualised using a five-way Venn diagram by the jquery.venny software (http://bioinfo.genotoul.fr/index.php?id=116) (Fig. 1). Such groups exactly match with those we described in a previous study (Ref. 17) in which HFF was investigated by a proteomic and bioinformatic approach, such as (i) inflammation/regulation of inflammation/acute phase, (ii) response to wounding, (iii) complement and coagulation cascades, (iv) protein–lipid complex/lipid metabolism and transport and (v) cytoskeleton organisation.

Inflammation/acute phase and *response to wounding* are the most representative classes including,

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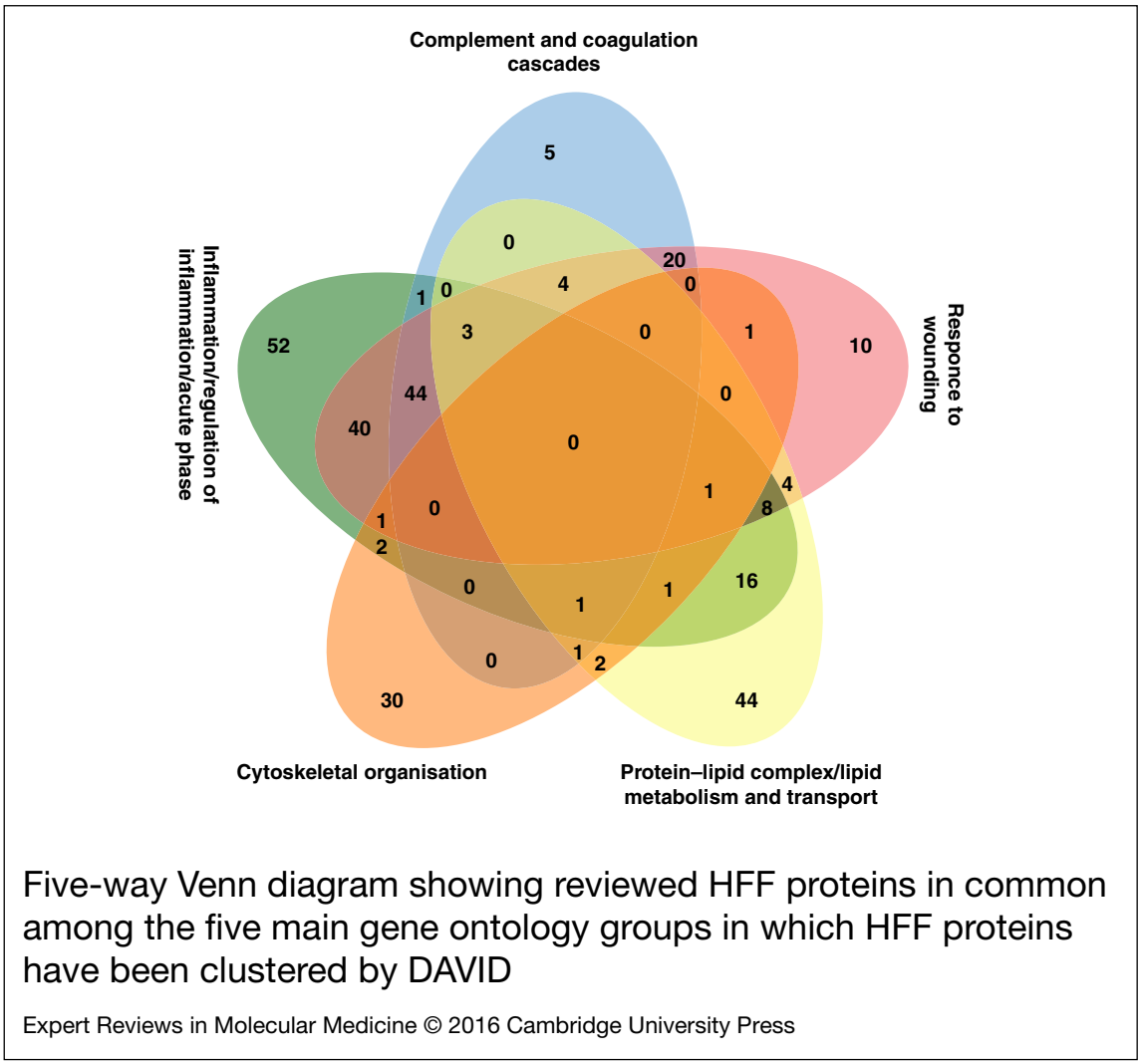

FIGURE 1.

Five-way Venn diagram showing reviewed HFF proteins in common among the five main gene ontology groups in which HFF proteins have been clustered by DAVID.

respectively, 172 and 136 proteins, that are 28 and 22% of the HFF proteins. Anyway, this is not surprising. Mammalian ovulation is actually assimilated to an acute inflammatory reaction, culminating with the release of mature oocyte, which is followed by coagulation and tissue repair processes to support corpus luteum formation (Refs 9, 14, 18).

In this regard, by DAVID analysis we pointed out that 72 proteins out of the 83 factors that were clustered in the *complement and coagulation cascades* are known to be active also in *inflammation and/or wound response* (Fig. 1). Therefore, several inflammation, coagulation and tissue repair active proteins are profoundly integrated in the follicle microenvironment, suggesting that balanced reactions among these proteins regulate physiological ovarian functions.

About 13% of HFF proteins we found out in Literature are involved in *lipid transport and metabolism*: triglycerides, cholesterol esters, phospholipids and nonesterified fatty acids are key molecules in a number of follicle processes, including hormonal responses and oocyte competence acquiring (Refs 13, 19, 20). Moreover, approximately half of the proteins belonging to this group interact with inflammation/acute phase, response to wounding, and coagulation groups (as detailed in Supplementary Table II and visualised in the Venn diagram, Fig. 1). In line with these results, well known inflammatory cytokines that control the immune system and the inflammatory response are reported to be able to modulate glucose and lipid metabolism, namely the adipokines. Several of them have been detected in the HFF, such as: IL-6, Tumor necrosis factor (TNF)-α, plasminogen activator inhibitor-1 (PAI-1), retinol-binding protein 4 (RBP4), C-reactive protein, etc. Furthermore, TNF-α and IL-6, as well as other cytokines, also play key roles in the activation of coagulation (Ref. 21). Interestingly, a number of pro- and anti-inflammatory molecules, active in immune cell trafficking and signalling as well in TNF-α activation and prostaglandin synthesis, have been proved to be expressed by theca, granulosa, and/or luteal cells and, in some measure, even by the oocyte (Refs 7, 22, 23).

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Finally, about 7% of HFF proteins, which were found out by our revision process, are involved in *cytoskeleton organisation*. Their presence into collected fluids may be because of a certain level of HFF contamination by follicular cells in consequence of physiological cell rupture during the pre-ovulatory phase and of cell damage caused by medical-procedure for oocyte retrieval.

In conclusion, about 24% of known HFF proteins are active in more than one of the above described four main clusters. These shared factors operate as functional 'molecular-bridge' among the delineated functional-groups, and their altered expression in HFF may profoundly interfere with fertility and assisted reproduction technology outcome. Moreover, such a tight cluster intersection underlines how the reproductive axis is subjected to systemic and follicular highly balanced integration of energy metabolism, inflammation, and tissue injury and repair.

Qualitative and/or quantitative variations of those factors, even if heightened by the hyperstimulation treatment and oocyte retrieval procedure, may impact on follicle and oocyte development and maturation in IVF programs thus justifying their evaluation as biomarkers for oocyte quality estimation (Refs 16, 17). Moreover, some HFF factors may quantitatively change in consequence of different responsiveness of individual patients to hyperstimulation protocols and/or to different causes of infertility. This may be of valuable impact not only on the assessment of oocyte quality, but also on individualised treatment planning for the eventual second cycle of ovarian stimulation.

The complexity of biological interactions occurring among HFF proteins and, in particular, their functional hierarchy have to be clarified in order to decipher how HFF multifunctional factors reciprocally interact and operate into the follicle microenvironment. We attempted to achieve this result applying pathway analysis.

Pathway analysis of HFF proteins

A total of 507 out of the 617 HFF unique proteins were unambiguously supported by the MetaCore network building tool v. 6.9. Their functional processing, by the 'direct interactions' algorithm, generated a statistically significant net of 315 elements reciprocally cross-linked by 776 interconnections (Fig. 2). To properly understand the generated net, MetaCore synonyms (applied in the net to name nodes) are reported in Supplementary Table I along with the conventionally accepted gene nomenclature. We also performed pathway analysis applying the 'shortest path' algorithm. In this analysis, the program introduces into the net specific molecular factors, which are not listed by the user among proteins to be processed but that are required, according to literature, to cross-link proteins under investigation. By this approach, the statistically significant generated net includes 370 proteins that are linked by 938 interconnections (Fig. 3). However, the core of both direct and shortest path networks integrate approximately the same number of proteins (301 versus 303) and share the same main central hubs, underlining limited variations occurring between them.

Functional pathways, in both nets, essentially converge on matrix metalloproteinases (MMPs), thrombin and micronutrient receptors, in particular vitamin D receptor and retinoid X receptor-alpha heterodimer (VDR/RXR-α) in the shortest path network. These proteins were the principal central hubs of the generated networks. Since the highest complexity of functional-interconnections was generated by the shortest path algorithm application, here we merely investigate the shortest path net.

MMP system in modification and reorganisation of the extracellular matrix (ECM)

The ECM is a highly complex and dynamic mesh of fibrous proteins and glycosaminoglycans. Its degradation and remodelling requires the combined action of several hydrolases, and correlated inhibitors, with different substrate specificities. MMPs are key enzymes in such processes, degrading several ECM and non-ECM components (Ref. 24). The MMP system, along with the fibrinolytic one, is actually retained the main proteolytic systems in ECM dynamics.

MMPs are mainly synthesised, as ProMMP precursors, by macrophages, mast cells and neutrophils. They are proteolytically activated by intracellular furin or by other MMPs and serine proteases, such as plasmin, thrombin, and neutrophil elastase in the extracellular milieu (Refs 25, 26, 27). ProMMPs may be activated also by conformational changes, without proteolytic processing, that are induced by chemical agents, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Ref. 28).

MMPs are the principal nodes of the network where the gelatinases MMP-2 and MMP-9 represent the two main central hubs (Fig. 3). MMP-2 and MMP-9 respectively establish 95 and 84 distinct interconnections with other net-molecules, which represent more than the 40% of HFF proteins entered into the net. The majority of edges occurring among gelatinases and other HFF proteins consists in 'outgoing' connections: from MMPs toward fluid components. Hence, gelatinases impact on ovarian physiology by a direct positive or negative control on HFF factors while, in turn, they are regulated by a very small number of proteins, several of which are other MMPs (Fig. 3).

Also stromelysin-1 (MMP-3) as well as, MMP-1, -25, -13 and -12, with even fewer edges, occur as central hubs of the network (Fig. 3). Several of the proteins cross-linked to these proteases are in common with MMP-2 and MMP-9; hence, the fraction of fluid proteins directly linked to MMPs slightly increase, reaching about 45% of net factors (Fig. 3).

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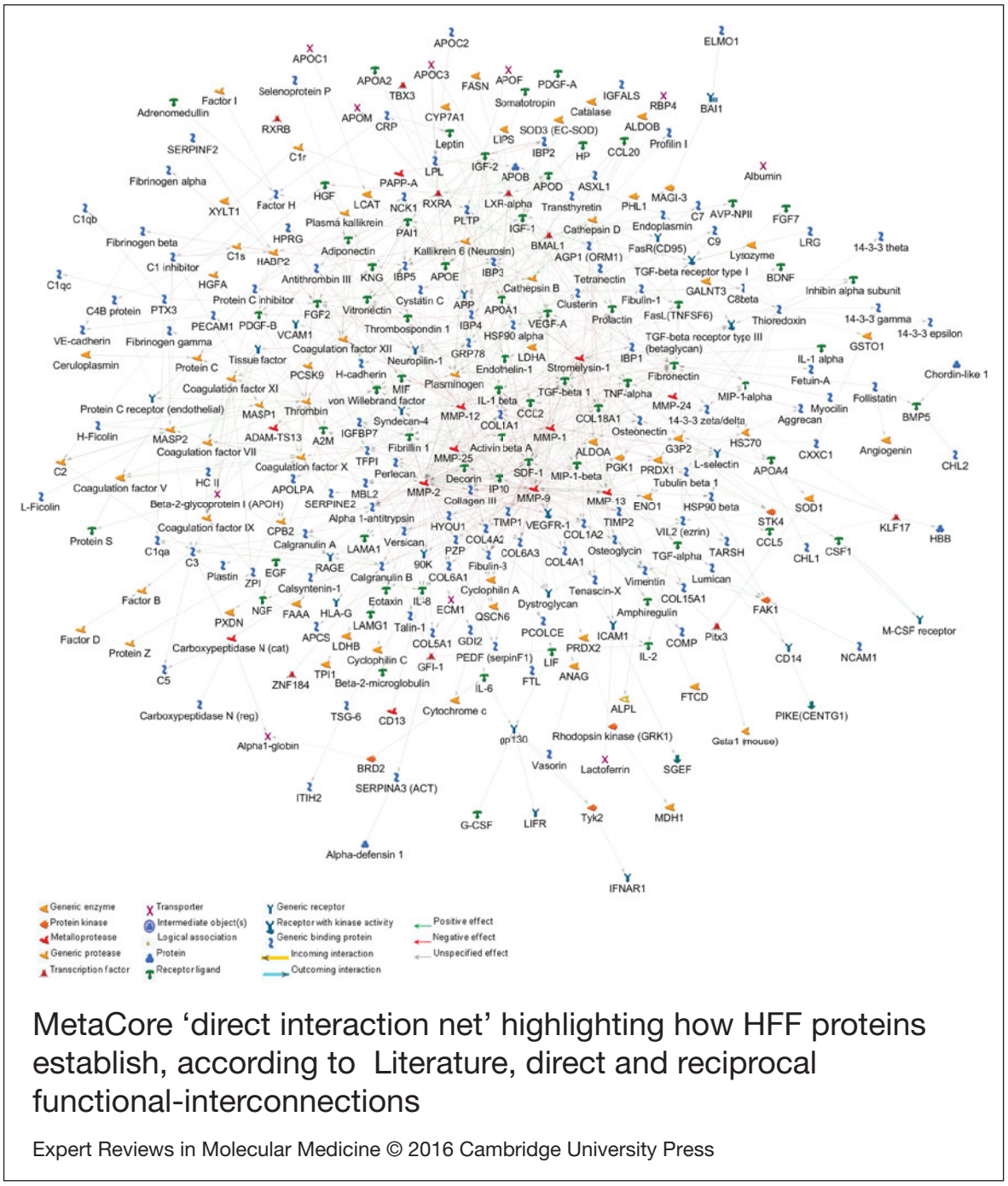

MetaCore 'direct interaction net' highlighting how HFF proteins establish, according to Literature, direct and reciprocal functional-interconnections

FIGURE 2.

MetaCore 'direct interaction net' highlighting how HFF proteins establish, according to literature, direct and reciprocal functional-interconnections. Network proteins are visualised by proper symbols, which specify the functional nature of the protein (network legend). Edges define the relationships existing between individual proteins, and arrowheads represent the direction of interactions.

In line with our pathway analysis data, MMPs have been reported as key proteins in follicle bioactivities triggering proteolytic cascades throughout development, maturation, apex rupture, corpus luteum formation and regression, and follicle atresia (Table 1) (Refs 29, 30). Accordingly, MMPs failure or reduced activity has been called into question for ECM derangements in subfertility and infertility, for aberrant follicle growth and deficiencies in ovulation (Refs 29, 31, 32, 33, 34, 35, 36). Controlling ECM organisation, MMPs are actually involved in the bioavailability control of hormones, growth factors, and osmotically active molecules by regulating local accumulation and activation of these factors, as well as cell response to extracellular stimuli and cell to cell communications (Refs 13, 37, 38, 39, 40, 41). As a result, MMPs 'escaped from control' may have severe negative effects on follicular and oocyte development and maturation causing excessive degradation not only of the follicular ECM, but also of resident growth factors

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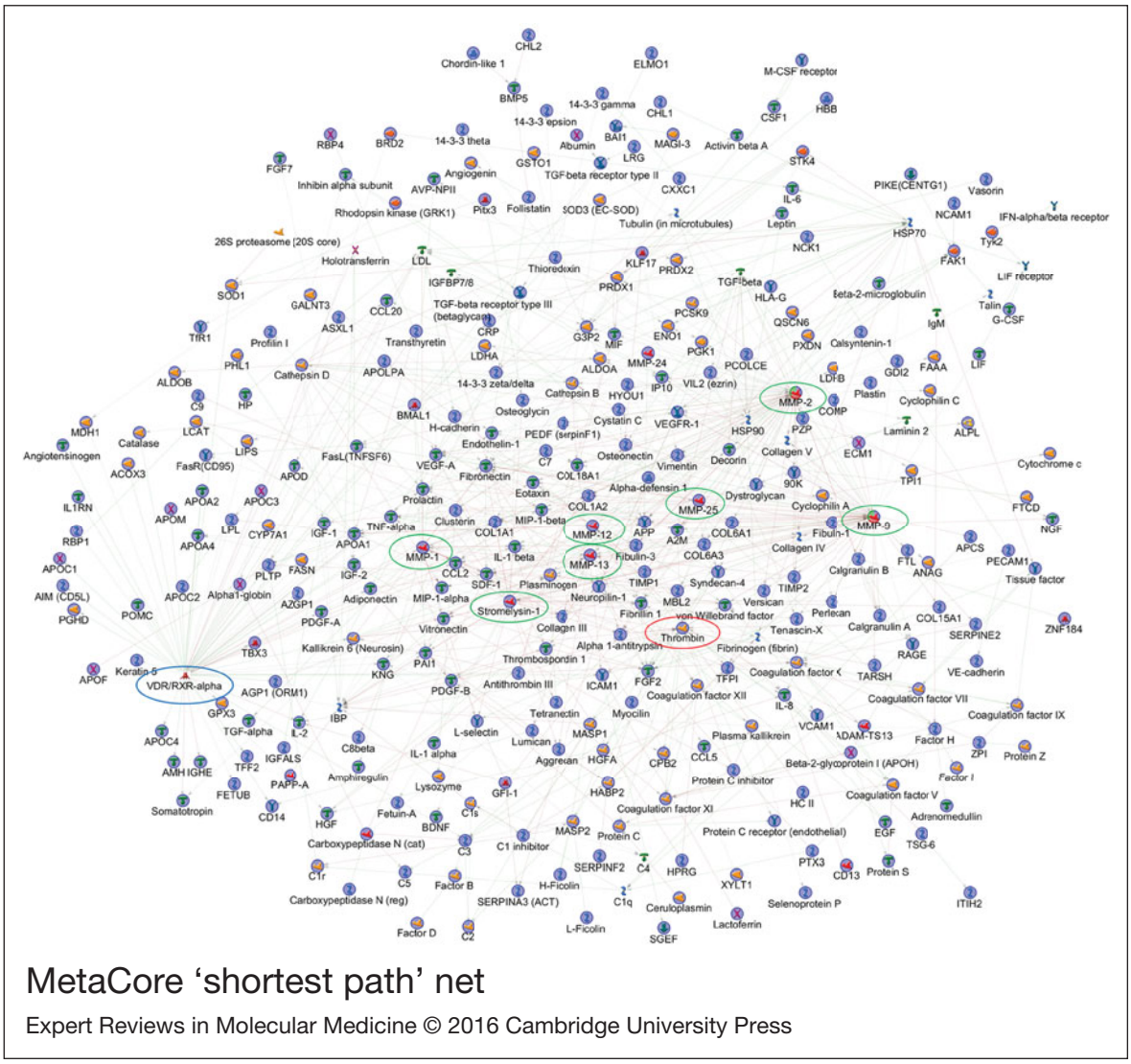

FIGURE 3.

MetaCore 'shortest path' net. The main central hubs are circled: MMPs (green), Thrombin (red) and VDR/RXR-α (light blue). To distinguish the functional factors added by the program, reviewed HFF proteins are encircle in blue. Several net nodes are collapsed in a representing protein used by the software to group two or more proteins linked by logical relations or physical interactions. Thus representing factors are marked by yellow little star attached to the factor visualising symbol. For network legend see Figure 2.

and co-factors, as well as cell-surface receptors on follicle somatic cells and, possibly, on the growing oocyte, too (Ref. 24).

MMPs aberrant activity may also impact on cumulus–oocyte complex (COC) and zona pellucida (ZP) integrity. Based on the tight metabolic dependence of the growing oocyte from cumulus cells as well as on the firm control that the oocyte exerts on cumulus cells physiology (Ref. 42), functional integrity of COC gap junctions is fundamental for oocyte development and maturation. MMPs 'uncontrolled' excessive activity may hence lead to the degradation of intra-cumulus and cumulus/oocyte junctions, with consequent impairment of oocyte maturation and competence acquiring. The access of MMPs to the ZP may also result in its partial degradation thus affecting sperm–oocyte interaction and fertilisation.

The selective proteolytic activity of MMPs is usually balanced by endogenous inhibitors alpha(2)-macroglobulins, and by tissue inhibitors of metalloproteinases (TIMPs) (Refs 43, 44). TIMP altered expression results in deregulation of ECM degradation and turn-over, but it may also impact on ovarian cell proliferation, differentiation and vascularisation during folliculogenesis (Ref. 34). Literature data proved TIMP production by theca and granulosa cells, ovary surface epithelium, corpora lutea, blood vessels and even by the oocyte (Refs 45, 46, 47). In this respect, a down-regulation of TIMP-1 may have a detrimental effect in the ovulation and an altered MMPs/TIMPs ratio may lead to a precocious regression of the corpus luteum with consequent depletion in progesterone and estradiol synthesis, thus to reduce the negative feedback to LH pituitary secretion (Ref. 36).

The inhibitory activity of TIMPs, is also decreased by peroxynitrites, which are generated by nitric oxide (NO) reaction with superoxide anion radical (O_2^-)

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TABLE 1.
MAIN ACTIVITIES AND PROCESSES IN WHICH METACORE CENTRAL HUBS ARE INVOLVED IN HUMAN FOLLICULAR FLUID

MMPs	
Systemic	• ECM degradation and remodelling; • Control of dynamics of osmotically active molecules, hormones, cytokines and growth factors and co-factors stored in the ECM; • Cleavage of cellular receptors (protease-activated receptors) and modulation of cellular adhesion, proliferation and apoptosis
In the ovary	• ECM degradation and remodelling; • Follicle development/maturation; • Apex rupture; • Corpus luteum formation/regression; • Follicle atresia; • Cumulus–oocyte complex integrity; • Zona pellucida integrity
THROMBIN	
Systemic	• Fibrinogen processing and platelet activation; • Cell proliferation; • Growth factors synthesis
In the ovary	• Corpus luteum formation/regression; • Oocyte maturation
VDR/RXR	
Systemic	• Regulation of genes coding for proteins involved in cell proliferation, differentiation, apoptosis and angiogenesis
In the ovary	• Follicle development/maturation; • Inflammation; • Ageing; • Control of AGE activity by inducing sRAGE synthesis

(Ref. 48). RNS as well as ROS have been described to be active in folliculogenesis, designation of the dominant follicle, meiosis progression, ovulation, as well as corpus luteum formation and regression (Ref. 49). NO is known to act in follicular maturation and in controlling blood flow of the follicle (Refs 50, 51). Here, it is produced by theca, granulosa/luteal, and capillary endothelial cells and significant high levels of NO have been detected in HFF (Ref. 51). NO and ROS 'acceptable' threshold values are index of healthy metabolic state of the preovulatory follicle and they may positively impact on IVF outcome (Ref. 52). On the contrary, inappropriate levels of ROS/RNS or inadequate total antioxidant capacity in ovary and follicle, have been suggested to adversely influence female fertility (Refs 49, 53, 54). In several tissues, RNS and ROS have cytotoxic effects determining membrane protein oxidation, lipid peroxidation and DNA damage (Ref. 55). Interestingly, in other biological systems, some evidences identify in ROS-dependent lipid peroxidation a 'cofactor' of MMP-1, MMP-2 and MMP-9 up-regulation (Refs 10, 56). Moreover, intracellular activation of MMP-2 by reactive oxygen/nitrogen species and consequent intracellular protein digestion has recently been elucidated (Refs 57, 58). Being MMP-2 constitutively expressed in the follicle, a similar modulation of MMP activity may also occur in the ovary, with some implications in reproduction failure. Consistent ROS levels in HFF have been correlated to decreased fertilisation, embryo quality and pregnancy rate (Refs 59, 60, 61).

ECM remodelling and complement/coagulation, response to wounding and inflammation pathways are functionally integrated by thrombin

Thrombin, as properly stressed by our network analysis, has emerged as a basic integration factor in HFF operating, at various levels, in the complement/coagulation system, response to wounding and inflammation (Refs 62, 63, 64), as well as in ECM remodelling by inducing expression and activation of MMPs (Refs 65, 66, 67, 68) (Fig. 4). Accordingly, thrombin resulted, with its 29 interconnections, one of the main central hubs of the generated net (Fig. 3).

Thrombin acts a key role in coagulation cascade by processing fibrinogen to generate fibrin and being the most potent platelet activator. However, thrombin is mainly generated after clotting and it has been indeed hypothesised to operate also after coagulation during tissue repair. It actually control even indirectly, through MMPs, the fibrinolytic process and regulate fibrin invasion, as proved in animal models, thus contributing to healing processes (Refs 65, 69, 70). By protease-activated receptor (PAR) signalling, thrombin induces proliferation, expression of growth factors, and ECM deposition (Ref. 71). Thrombin indeed modulates ECM-remodelling determining matrix degradation by MMP activation, but also inducing ECM formation. Accordingly, it may have implications in corpus luteum organisation and in IVF outcome. To date, fibrinolysis impairment has been correlated to implantation failure and recurrent pregnancy loss (Ref. 72).

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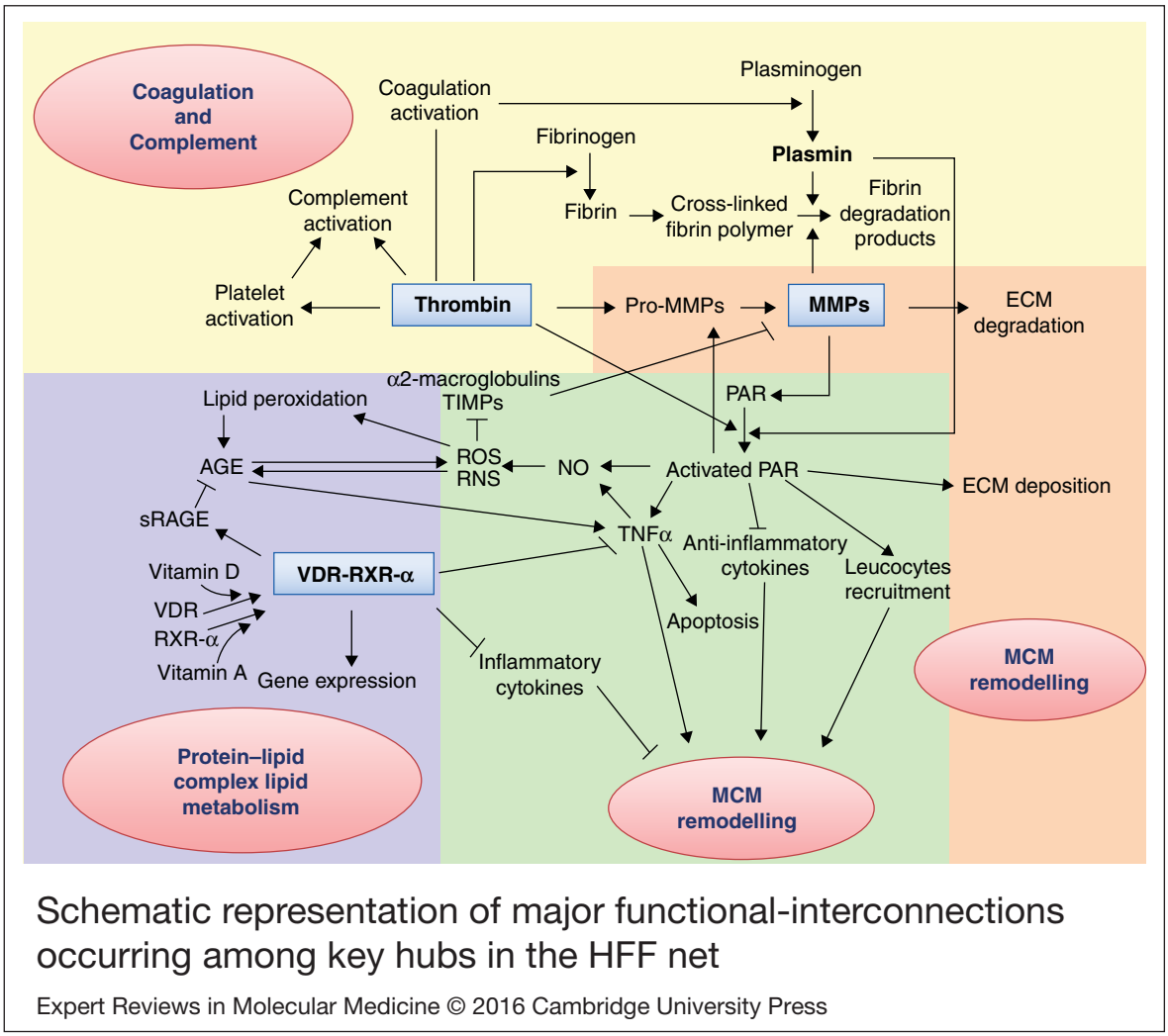

FIGURE 4.

Schematic representation of major functional-interconnections occurring among key hubs in the HFF net. The schema provides a direct and simplified overview on the complex crosstalk exerted by MMPs, Thrombin and VDR/RXR-α in controlling, modulating and balancing inflammation, coagulation, healing process and lipid metabolism in the follicular microenvironment, as highlighted by pathway analysis.

Thrombin may influence reproductive performance also because of its involvement in regulation of inflammation. Theca, granulosa, and cumulus cells are, through PARs, thrombin responsive (Refs 73, 74, 75). In target cells, proteolytic activation of PAR receptors results in pro-inflammatory cytokine secretion (e.g. IL-1, IL-6, IL-8 and TNF-α), anti-inflammatory cytokine down-regulation, and in recruitment of leucocytes also by monocyte chemotactic protein-1 (MCP-1) production (Refs 63, 76, 77). A proper time-dependent and balanced activity of cytokines is absolutely required in physiological maturation and rupture of the follicle. On the other hand, aberrant cytokines activity may profoundly affect follicle dynamics, as in the case of TNF-α. Elevated intra-follicular TNF-α concentrations have been correlated to reduced development and inhibited maturation of the oocyte as well as to decreased embryo competence and quality (Refs 78, 79). TNF-α can also induce apoptosis and follicular atresia in presence of proper receptors, specific for the apoptotic pathway, that are exposed on granulosa cells and even on the oocyte plasmalemma (Refs 80, 81). Moreover, TNF-α also controls NO concentration by the induction of NO synthase 2 activity (Refs 82, 83). High levels of NO in HFF have been associated to embryo lower grading and reduced implantation and pregnancy rate (Ref. 84). Finally, TNF-α and ILs induce MMP expression and activation (Refs 24, 85, 86). While proper-operative MMP pathways are crucial in the ovary, MMP excessive activity negatively correlates, as above stated, with reproductive potential.

In conclusion, as highlighted by our functional analysis, Thrombin absolves key functions in folliculogenesis by performing, controlling and triggering several biochemical and molecular processes that are known to be essential in the physiological functioning of the ovary. Indeed, thrombin dysregulation, with consequent detrimental effects on inflammation, coagulation

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and healing process balancing, may negatively impact on fertility.

Micronutrients: key factors in follicular central processes

According to the DAVID cluster analysis, proteins that are active in lipid metabolism constitute one of the major classes in HFF. In the net, several of those factors are centred in the VDR/RXR-α receptor complex, hence suggesting the existence of a close functional correlation among proteins involved in lipid metabolism and micronutrients. VDR/RXR-α receptors belong to the steroid/thyroid hormone/retinoid nuclear receptors (NRs) super-family and their heterodimerisation regulates the expression of genes coding for proteins involved in numerous cellular functions, such as proliferation, differentiation, apoptosis and angiogenesis. Intriguingly, as highlighted by our pathway analysis, VDR/RXR-α directly or indirectly controls MMPs/TIMPs balancing, the plasminolytic, fibrinolytic and cathepsin/cystatin systems, vasodilatation, interleukins, TNF-α and complement activity (Refs 87, 88, 89, 90, 91) (Fig. 4). Such a high functional integration of the VDR/RXR-α dimer in follicular dynamics has been further clarified visualising the shortest-path net according to the 'subcellular localisation' of entered nodes, as shown in Figure 5. Here, the VDR/RXR-α operates as a molecular 'puppeteer' that directly or indirectly controls several cellular and extracellular proteins in the follicle. Indeed, vitamins D and A, emerge to have a pivotal role in follicular processes influencing reproductive performance (Refs 92, 93, 94, 95).

Vitamin A is a generic term to indicate a large class of related compounds that cannot be de novo synthesised by animals and whose intake can occur only through nutrition. Retinoic acid (RA) is vitamin A active form that controls gene transcription through specific NRs: all-*trans*-RA binds retinoic acid receptor (RAR-α, β and γ) and 9-*cis*-RA binds retinoid X receptor (RXR-α, β and γ) (Ref. 62). Unliganded RXR may dimerise not only with RAR, but also with thyroid hormone receptors (THR) and with VDR (Ref. 62). Liganded RXR is instead a heterodimeric partner of other NRs, such as peroxisome proliferator-activated receptor (PPAR) and liver-X receptor (LXR), both involved in lipid metabolism and homeostasis (Refs 96, 97).

Vitamin D is a secosteroid hormone whose precursor is an intermediate of the cholesterol metabolism. Its active form, the 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), also known as vit D3, interacts with the NR VDR to perform its own functions. VDR has been hypothesised to control several human genes, even up to 5% of the entire genome (Refs 98, 99). Consequently, vitamin D aberrant endogenous production or dietary up-take may profoundly influence the physiological state of the organism. Besides its well-known involvement in calcium and phosphorus homoeostasis, vitamin D has been suggested to influence physiological processes in several different tissues, including male and female reproductive systems (Refs 100, 101).

In addition, vitamin D supplementation has been recently demonstrated to increase the serum soluble form of receptor for advanced glycation end-products (sRAGE) (Ref. 102). These soluble receptors exert a 'scavenger' activity for the advanced glycation end-products (AGEs), which are potent cytotoxic metabolites. sRAGE have been also detected in HFF and their increased concentration has been associated to positive IVF outcomes (Refs 103, 104, 105). Thus, by inducing the expression of sRAGE through VDR/RXR-α, vitamin D may counterbalance the AGE/RAGE system. Actually, the scavenger activity of sRAGE reduces AGEs detrimental effects by preventing their interaction with cellular specific receptors (cRAGEs), which are included in all the generated nets (RAGE node in Figs 2, 3 and 5) (Refs 106, 107).

AGEs are implicated in inflammation, ovarian aging, PCOS pathogenesis, and metabolic syndrome effects (Refs 107, 108, 109, 110, 111). They are potent pro-inflammatory molecules that cause the generation of ROS along with an enhanced expression of IL-1, IL-6 and TNF-α in macrophages (Ref. 112). Ovary has interestingly emerged to be a target tissue of AGE deposition and the interaction of these compounds with cRAGE, expressed by theca and granulosa cells (Ref. 113), determine cell dysfunctions and anomalous follicular growth (Ref. 114). The intrafollicular accumulation of AGEs was described to negatively impact on embryo growth and pregnancy rate (Ref. 115).

Conclusions and future perspectives

Oocyte quality estimation during ART procedures is so far mainly based on morphological criteria that are considered largely unsatisfactory. Even if several supposed biomarkers have been identified, the outcomes of IVF procedures remain poor because of the limited improvements in clinical treatment strategy. In fact, despite fundamental progresses of basic research, translational medicine still requires lots of effort in order to translate new discoveries into helpful clinical applications. The scientific community is therefore focused on the research of other approaches to this issue based on genomic, transcriptomic and proteomic molecular investigations about ovarian follicle components, including somatic cells and follicular fluid.

All these are noninvasive approaches and, thus, may result the best strategy to identify molecular biomarkers directly related to oocyte development and competence without affecting gamete health.

Nevertheless, OMICs analyses provide at once a huge amount of biological information but often they do not infer clear functional relationships among identified molecular effectors, and in some cases obtained data are not original.

In silico functional analyses of HFF proteins, which were selected from Literature according to specific

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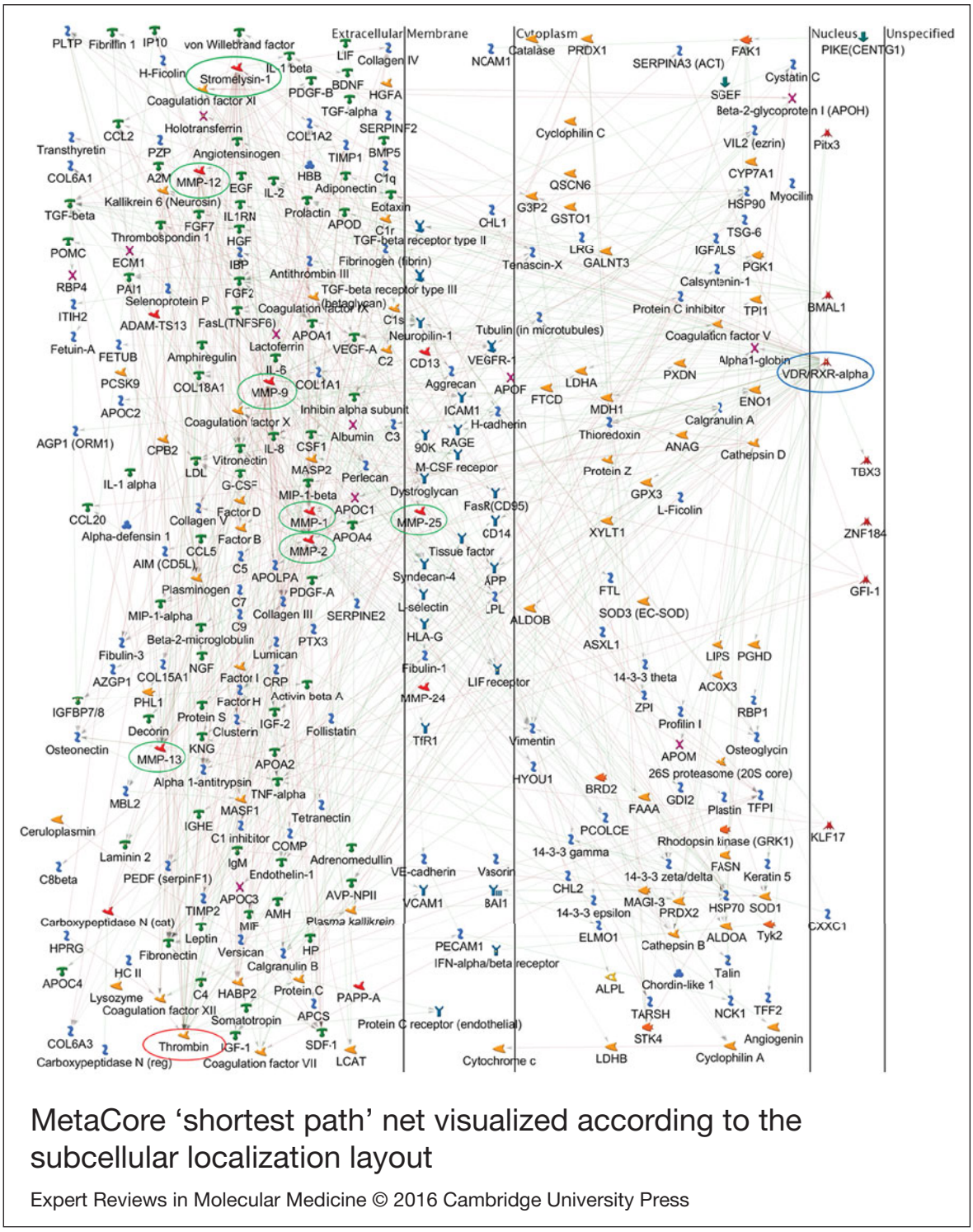

FIGURE 5.

MetaCore 'shortest path' net visualised according to the subcellular localisation layout. VDR/RXR-α, which is cycled in light blue, evidently emerges as a key transcriptional factor with pivotal role in controlling numerous downstream proteins. MMPs and Thrombin are highlighted in green and red, respectively. For network legend see Figure 2.

criteria, enabled us to provide a broad overview on functional characteristics and dynamics of follicle microenvironment that was, to our knowledge, never attempted before. We actually did not limit our analysis to the functional processing of HFF proteins, but we also tried to 'summarise' the several experimental reports on HFF protein composition. Combining results from different investigations, we indeed obtained the largest up to date written list of HFF proteins whose presence in the fluid was experimentally observed and unambiguously reported. Proteins that were merely supposed to be present in HFF, according

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to homology or to detection of the corresponding mRNA, were actually excluded by our study.

The innovative results we achieved lead to the delineation of a really complex and integrated protein framework in which the oocyte acquires competence. Pathway analysis outlined key proteins with fundamental roles in follicular physiology. These are the central hubs of the main molecular pathways active in the HFF milieu, namely: *MMPs, thrombin* and *vitamin A* and *D receptors*.

In regard to MMPs, biological and functional integrity of 'follicle matrixes' is essential to ensure proper folliculogenesis, oocyte development and competence acquiring, ovulation, oocyte passage through the oviduct, fertilisation and implantation. Altered deposition, remodelling, end degradation of the follicular ECM, even depending on different causes and effectors, have detrimental consequences on female reproductive potential. Combined dynamics of several different HFF factors, with described correlations to subfertility or infertility, mainly converge or depend on HFF MMP activity and therefore they suggest these hydrolyses as possible indicators of follicular physiology and oocyte quality. Interestingly, thrombin exerts a pivotal rule in controlling, modulating and balancing inflammation, coagulation and healing processes, also by regulating MMP activity.

MMPs, and in particular gelatinases, but, to some extent, also thrombin may be precious pharmacological target to improve fecundity. In spite of this and according to their spectrum of action, MMPs and thrombin exogenous modulation has to be carefully evaluated in order to prevent deleterious side effects.

Finally, vitamins D and A emerge as key players in follicular central processes thus contributing to further bring into the limelight of reproductive-medicine interest healthy habits as one of the major factors able to influence, also in pathological conditions, the reproductive performance.

Even if more translational medicine efforts should be made to encourage standardised systematic biomarker validation studies in follicular milieu, the functional proteomic approach we applied may be a promising tool for oocyte quality estimation in order to improve the reproductive outcome.

Supplementary material

The supplementary material for this article can be found at http://dx.doi.org/10.1017/erm.2016.4.

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Conflict of interest

None.

Ethical standards

Not applicable.

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*Corresponding author:
Paola Piomboni,
Department of Molecular and Developmental Medicine,
University of Siena,
Viale Bracci 14, 53100 Siena, Italy.
E-mail: piomboni@unisi.it

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