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Doctoral Thesis

**EXPRESSION AND INTRACYTOPLASMIC
DISTRIBUTION OF STAUFEN AND CALRETICULIN IN
HUMAN MATURING OOCYTES**

(VET/01)

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1.INTRODUCTION

1.1 Oocyte maturation in the human

The human oocyte is certainly the most fascinating cell in our body for its peculiarities both in structure and in function. The egg acquires all its developmental potential during a long period of time compared to all other cells. Oogenesis begins early during embryonic-fetal life and continues well after birth through a process entailing first the production of numerous oogonia followed by a dramatic reduction in the pool of available oocytes that will be retained in the ovary. The oogonium is organized within the simultaneously growing female gonad generated in the fetus and becomes enclosed in the follicle where coordination of the forthcoming growth and maturation will take place. The gonad is generated from a single primordium of mesenchymal tissue following the invasion of three cell populations: primordial germ cells, epithelial cells and mesonephric germ cells. The first will lead to oogonia, the second to the follicular structure and the third giving rise to the theca and vascular system of the gonad. Ovarian development ceases and waits for the organism to become hormonally competent to support complete oocyte maturation allowing the egg to achieve the

capability to undergo fertilization and give rise to an embryo able to develop and implant in the uterine cavity. The pool of oocytes that has the ability to perform this last step is small compared to the totality of germ cells and the initial reduction of this gametic heritage is known under the name of follicular atresia (Johnson, 2008). The mammalian ovary contains a stock of resting follicles a number of which is everyday activated to enter in the growing phase. In fact follicles begin to form during fetal life but most of them remain in a resting stage until they either degenerate or enter, if appropriately stimulated, the growth period. In humans it can be proposed from both direct count (Forabosco *et al.*, 1991) and mathematical extrapolation (Gougeon *et al.*, 1994) that each ovary at birth contains between 250.000 and 500.000 resting follicles. In all mammalian species, follicles leave the stock of resting follicles in a continuous stream, either by apoptosis or by entry in the growth phase, thus depleting the ovarian reserve with age. In humans, this phenomenon differs among subjects but in general it accelerates from approximately 38 years of age onward to arrive in menopause with less than 1000 resting follicles (Faddy *et al.*, 1992; Gougeon *et al.*, 1994)

1.2 Molecular and organelle reorganization during maturation

Mammalian oocytes acquire a series of competencies during follicular development that play critical roles at fertilization and subsequent stages of preimplantation embryonic development. Before the mammalian oocyte engages in the fertilization process, it must acquire an array of molecular and cellular assets defining its developmental potential. These properties specify competencies to complete meiosis and initiate mitosis, support monospermic fertilization and egg activation and ensure a timely transition from reliance of gene products of oocyte origin to those derived from the zygotic or embryonic genome (Albertini *et al.*, 2003).

Meiotic maturation requires the acquisition both of nuclear and cytoplasmic competence and this complex mechanism involves most of the organelles, the cytoskeleton and molecules that move from the nucleus to the cytoplasm.

Compared to somatic cells whose growth in volume is limited by the intervention of mitosis, the female gamete does not undergo cell division and therefore the oocyte continues to expand to 100 fold its volume reaching the diameter in the human species of 120 μm . Such an impressive growth aims to provide the egg with a sufficient amount of organelles and molecules that will support early stages of embryonic development up to the complete metabolic autonomy.

Meiotic maturation is a rather well established process in mammals however some mechanisms are not yet completely known in humans given the difficulties in obtaining oocytes for research purposes (Van Steirtegem, 2003).

During their growth oocytes transcribe genes and translate proteins so that the accumulation of maternal factors occurring during this crucial phase is essential for the activation of the embryonic genome and successful preimplantation development (Dean, 2003)

Fertilization in mammals assembles two "silent" genomes that have developed separately during gametogenesis in maternal and paternal bodies. Therefore, the first activity of the embryo that results is that of constituting a single and functional genome able to continue autonomously during the subsequent stages of development. The first requirement is thus to loose the tight chromatin structure that compact and silence the gamete genome and subsequently to obtain (as happens in somatic cells) an embryo chromatin remodelling to make gene expression possible. These modifications of chromatin structure (which are different in the maternal and paternal individuals) require specific factors present in the cytoplasm of the oocyte.

The early stage of development is, therefore, that unique moment during which the individual is "governed" by the genetic information synthesized from the genome of another individual (the mother) and accumulated during the process of oogenesis either as proteins or RNA

in the cytoplasm of a single cell (the oocyte). The oogenesis, itself, is characterized by intense synthesis of RNA and proteins, during the growth phase of the oocyte, which can be stored for very long periods of time (months or years) and it is evident that a period of storage so long requires specific molecular processes. The mRNA in fact must be protected from the translation mechanism and the proteins must be accumulated in a non-functional form. The collection of all this information also requires the existence of specific mechanisms that are needed in the post-transcriptional phase with respect to the mRNA and in the post-translational one regarding the proteins: this system allows appropriate molecules to be available at the time requested.

The oocyte maturation marks the beginning of this phase of activation of maternal transcripts and proteins and involves the "unmasking" of maternal mRNA concomitantly to post-translational changes of stored proteins. This process loosens the stabilization mechanisms that had been imposed during oogenesis. At the end of this route maternal information will be available to the developing embryo (Duranthon and Renard, 2003).

In the process of transport of mRNAs from the nucleus to the cytoplasm of the cell a wide number of proteins and factors are involved. One of these is represented by *Staufen*, whose function is to bind the mature mRNA and transport it to specific sites of the cytoskeleton, where it

promotes the anchorage for the translation of the corresponding protein (Wickham *et al.*, 1999).

There are several studies on the characterization of *Staufen* in oocytes from various species such as murine (Saunders *et al.*, 2000) bovine (Calder *et al.*, 2008) and porcine (Brevini *et al.*, 2007a) but there is little information in humans.

The production of proteins by the cell comes from the information contained in its DNA. This process consists of two stages: the transcription of the gene coding for the protein in a strand of messenger RNA (mRNA) and the translation of the latter into a protein. In eukaryotic cells these processes take place in separate compartments: the first in the nucleus, the second in the cytoplasm.

The mRNA must, therefore, be transported from the cell nucleus to the cytoplasm through the nuclear pores. This transfer is highly selective and it means that "to be ready for export" an mRNA must achieve a number of specific modifications (mature mRNA) and must be linked to the appropriate set of proteins.

Superimposed to gene expression regulatory mechanisms, the nucleus as an organelle undergoes significant positional rearrangements during maturation. During this process at both MI and MII, the spindle is localized cortically just beneath the oolemma. This is obviously imposed by the necessity to direct a cleavage plane suitably near the cell surface in order to extrude a small polar body and minimize the loss from the

oocyte environment of organelles (e.g. mitochondria) and regulative molecules (e.g. γ -tubulin) from which the development of the ensuing embryo will depend crucially (Barrett and Albertini, 2007). The way in which spindle asymmetry is obtained has been extensively studied in the mouse. In this species, it has been repeatedly reported that, after recovery from large antral follicles and removal of cumulus cells, fully-grown oocytes display the GV in a central or peri-centric position and that germinal vesicle breakdown (GVBD) occurs in the same position (Fabritius *et al.*, 2011) (Chaigne *et al.*, 2012). This implies that the microtubular dynamics leading to the formation of the MI spindle also requires a process of repositioning from the centre to the periphery. Once formed after breakdown of the GV, the MI spindle in fact appears to migrate centripetally in a pole-first orientation at a speed of 0.12 $\mu\text{m}/\text{min}$ (Verlhac *et al.*, 2000). Cortical domains pre-determined to host the spindle do not seem to exist because, after experimental displacement, the spindle is moved again by cytoskeletal forces towards the nearest cortical region (Schuh and Ellenberg, 2008). Oocytes treated with nocodazole, a microtubule depolymerising agent, are still able to support MI translocation (Verlhac *et al.*, 2000), while treatments that depolymerise (Verlhac *et al.*, 2000), stabilize (Li *et al.*, 2008), or interfere (Li *et al.*, 2008) with the regulation of actin filaments can inhibit spindle migration. Therefore, actin undoubtedly plays an essential role in this process, although it is less clear how.

in the mature oocyte, cytosolic oscillations in the concentration of free calcium ion (Ca^{2+}) are fundamental to orchestrate the events (GC release, resumption of meiosis, pronuclear formation, recruitment of maternal RNAs) that collectively characterise the fertilization process. The ability to sustain intracellular Ca^{2+} oscillations is typical of and essential for MII oocytes but is not fully established in fully-grown GV stage oocytes (Jones *et al.*, 1995). Studies carried out in the mouse have clarified the cellular basis of such a difference between mature and immature oocytes. Ca^{2+} is stored in the cisternae of the endoplasmic reticulum (ER). At fertilization, its release is mediated by inositol 1,4,5-trisphosphate (IP3), which is generated from cleavage of the oolemma-bound lipid phosphatidyl-1,4-trisphosphate. Interaction of IP3 with its receptor situated in the membranes of the ER triggers the release of Ca^{2+} in the cytoplasm (Runft *et al.*, 2002). This is made possible by the fact that ER development and receptor sensitivity to IP3 increase during maturation and are maximal in the mature oocyte (Mehlmann *et al.*, 1996). In GV-stage mouse oocyte, the ER is continuous with the nuclear envelope and forms aggregate preferentially distributed in the oocyte interior (Mehlmann *et al.*, 1995). At GV breakdown, ER membranes organize around bundles of microtubules and envelope the newly formed MI spindle. Interestingly, in oocyte experimentally arrested at the GV stage, this ER rearrangement does not occur, suggesting dependence from cell cycle mechanisms. ER redistribution at GVBD is

also prevented by treatment with inhibitors of microtubule polymerisation or cytoplasmic dynein (FitzHarris *et al.*, 2007). As maturation progresses further, the ER cisternae and vesicles develop and form aggregates of 1-2 μm occupying also the cortical cytoplasm, although in a polarized fashion involving a preferential accumulation in the vegetal hemisphere, i.e. opposite to the MII spindle position (Mehlmann *et al.*, 1995). This second phase of rearrangement is also dependent on cytoskeletal elements, as suggested by the fact that it does not occur in the presence of microfilaments depolymerizing agents (FitzHarris *et al.*, 2007). In coincidence with ER growth and redistribution, the IP3 receptors increase in number and become localized in the oocytes cortex (Mehlmann *et al.*, 1996), area from which they are excluded in immature oocytes. These modifications of the ER and IP3 receptor are believed to account for the augmented sensitivity of mature oocytes to Ca^{2+} -release mechanisms that regulate fertilization. In other mammalian species, the pattern of ER rearrangement during maturation may be different, as observed in the cow (Payne and Schatten, 2003) and hamster (Shiraishi *et al.*, 1995) where elements of the ER are found in a cortical position in GV-stage oocytes and more homogeneously dispersed in small clusters in mature oocytes. Human oocytes display a pattern of changes in ER distribution and IP3 abundance similar to the one of the mouse. In oocytes found at the GV-stage after controlled ovarian stimulation, the ER is in most

cases excluded from the cortical region and does not appear organized in cluster (Mann *et al.*, 2010). Unlike the mouse, though, ER accumulation is not visible around the GV. In in-vivo matured oocytes, ER elements are associated in clusters of 2-3 μm preferentially localized in the cortex, but also discernible in more internal regions. Therefore, also in the human, the ER undergoes significant changes during maturation in its distribution that reflect an enhanced ability to release Ca^{2+} .

All the above-described cellular rearrangements occurring during oocyte maturation are energetically demanding. Energy derives from cellular ATP as a result of mitochondrial activity. Mitochondria are themselves subject to redistribution and changes in activity during transition from the GV to the MII stage, in a fashion that can profoundly influence the quality of the mature oocyte and the destiny of the ensuing embryo. In fact, compromised oocyte quality is associated to aberrant mitochondrial rearrangement and low ATP levels (Van Blerkom *et al.*, 1995) (Van Blerkom, 2004). Recent studies in the mouse have unravelled the dynamics of these mitochondrial changes. During in vitro maturation, mitochondrial ATP undergo three distinct phases of increase in ATP production, separated by two shorter phases in which ATP returns to basal levels (Yu *et al.*, 2010). These phases of augmented activity correspond to GVBD, spindle migration and the transition between MI and MII. From previous observations it is known that on

large scale an accumulation of mitochondria surrounds the nucleus at the time of GVBD (Bavister and Squirrell, 2000), accompany the spindle during migration, and are present at the side of polar body emission. However, these rearrangements do not seem to be functional to or associated with the above-mentioned burst in ATP production, because when their formation is prevented by inhibitors of microtubule polymerisation, ATP changes still occur (Yu *et al.*, 2010). On the contrary, formation of mitochondrial clusters on a smaller scale appear to be a pre-requisite for an increase in ATP production. Formation of these small clusters is temporally co-ordinated with the burst of ATP observed at GVBD and the successive maturation steps. Mitochondrial clustering seems to be driven by the actin microfilaments network. This cytoskeletal array, in fact, is dissolved and re-formed in coincidence with the formation of small mitochondrial clusters and its disruption with cytochalasin B, which causes actin depolymerisation, prevents the formation of mitochondrial clusters and the increase in ATP production (Yu *et al.*, 2010). Therefore in the mouse, the cytoskeleton, and in particular the microfilament network, appear to direct mitochondrial redistribution on a small scale and ATP production in support of GVBD, spindle translocation and first polar body emission. However, little is known why mitochondrial clustering is required for an increase in ATP generation. Data indicating a strict correlation between mitochondrial distribution patterns and developmental ability have been obtained with

porcine oocytes. In the pig, mitochondria are restricted to the periphery of GV oocytes but migrate during maturation to the inner region of the cell both in vitro (Sun *et al.*, 2001b; Sun *et al.*, 2001c) and in vivo (Torner *et al.*, 2004). Even though there is paucity of information about the correlation between this phenomenon and subsequent oocyte development, it has been suggested that relocation of mitochondria occurs in oocytes with high developmental competence (Brevini *et al.*, 2007a).

The Golgi is composed of stacks of membrane-bound structures known as "cisternae" and this apparatus is specialized in modifying, sorting, and packaging macromolecules for cell secretion or intracellular needing. Although the role and function of the Golgi is rather well established during mitosis, the dynamic of Golgi membranes during meiosis is still partially unknown. Experiments conducted in rodents have shown that in GV stage oocytes the Golgi consists of a series of structures dispersed in the ooplasm, but considerably more concentrated in the interior than at the cortex. During in vitro maturation the large Golgi apparatus structures fragment at GVBD and disperse homogenously throughout the ooplasm, remaining in a fragmented state at the MII stage. Although protein secretion is blocked during meiotic maturation, Moreno *et al.* (Moreno *et al.*, 2002) have shown that brefeldin A (a drug that inhibits protein secretion by blocking membrane trafficking from the ER to the Golgi apparatus) is

able to reversibly inhibit the IVM of mouse oocytes and that this block occurs at the same stage at which it is arrested by protein synthesis inhibitors, suggesting that, in addition to protein synthesis, progression of murine oocyte maturation possibly also requires functional membrane trafficking sometime after GVBD, resulting in either the modification of proteins at the Golgi level, or the delivery of these proteins to appropriate (post-Golgi) sites. In 2012 Racedo and colleagues (Racedo *et al.*, 2012) demonstrated that, in in-vitro matured bovine oocytes, cytoplasmic maturation is accompanied by a series of dynamic changes of the Golgi involving an active microtubular function. Using different specific inhibitors these authors demonstrated that prior to GVBD, the Golgi moves from the centre of the cytoplasm to the cortical area. Afterwards, a second translocation occurs between GVBD and MI, repositioning the Golgi from the periphery to the central cytoplasmic area.

During the growth phase of oogenesis, at early stages of folliculogenesis, the Golgi complex is situated in internal regions of the oocyte cytoplasm and subsequently proliferates, becomes hypertrophic, and finally produces small vesicles that progressively acquire a more peripheral or cortical position. These vesicles coalesce, forming mature Golgi complexes in a continuous process during oogenesis that extends to the preovulatory period (Ducibella *et al.*, 1994). During the maturation phase, initiated by the resumption of meiosis and completed

by the achievement of the metaphase II (MII) stage, Golgi complexes are subject to redistribution assisted by cytoskeletal infrastructures. In particular, the peripheral relocation of GC during maturation appears to occur with the intervention of microfilaments (Sun *et al.*, 2001a). In mature oocytes of almost all mammals (with the exception of mouse (Nicosia *et al.*, 1977; Okada *et al.*, 1986), GC are homogeneously distributed cortically just beneath the oolemma (De Santis and Brevini, 2013)

1.3 The mechanism of Polyadenylation

Translational control of specific maternal mRNAs is critical during early development and changes in the translational activity of specific mRNAs often can be correlated with changes in the length of their poly(A) tails. In general, mRNAs that receive poly(A) become translationally active, whereas those that lose poly(A) become translationally quiescent. These correlations are widespread, occurring in many species. In higher eukaryotes mRNA 3'-end formation occurs by cleavage and polyadenylation as illustrated in the cartoon below (Figure 1). Cleavage and polyadenylation is directed by a poly(A) signal in the RNA.

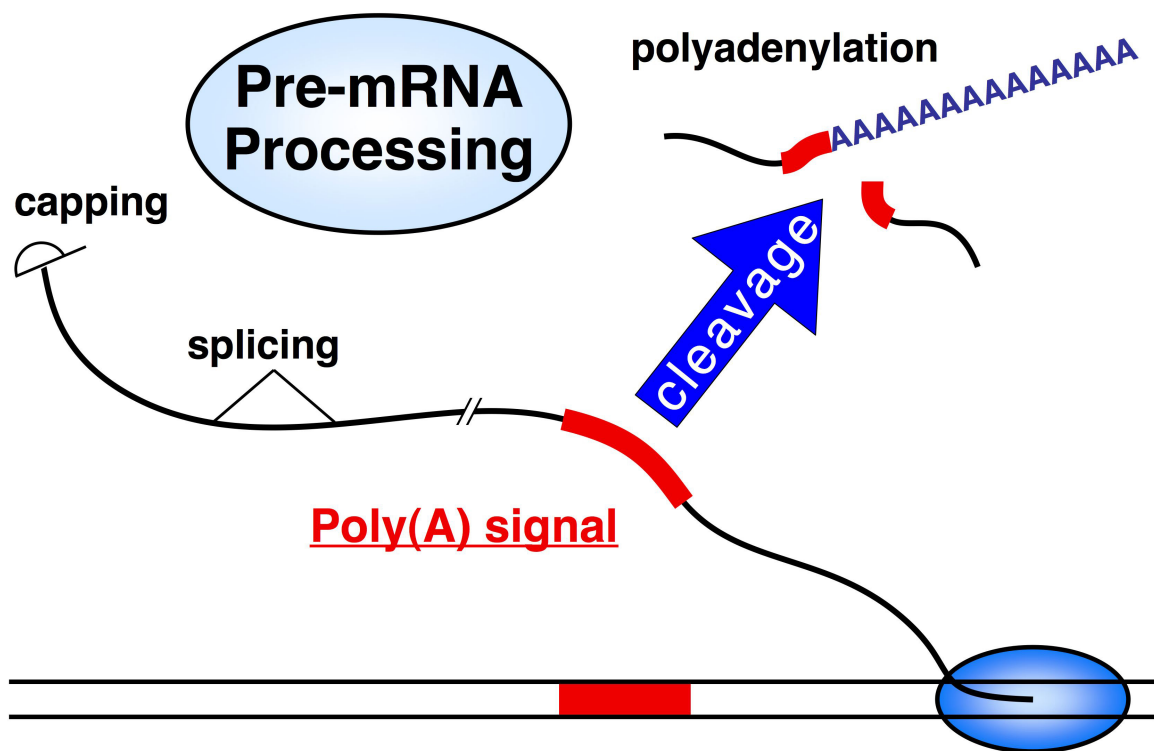


Figure 1: General scheme of Polyadenilation process

From: <http://www.biochemistry.ucla.edu/biochem/Faculty/Martinson/>

RNAs and proteins can be synthesised even several weeks before they are used and specific mechanisms exist in order to optimise their storage in a quiescent form and to ensure their availability at the right time during oocyte maturation and early embryonic development. Oocyte quality and its ability to sustain embryonic development largely depend on the efficiency of such storing process, as well as on the correct and timely reactivation of the stored molecules (Brevini *et al.*, 2007b). The half-life of individual mRNAs may depend on their degradation by specific exonucleases. For this reason, RNA molecules

are protected from attack by these enzymes by a specific structure located at the 5' region of the transcript, termed the methylguanosine cap structure (Shatkin and Manley, 2000). The latter interacts with the 3' tail of adenosine residues (3'-AAAAAAAA), in controlling mRNA stability and decay (Proudfoot, 2000). Namely, shortening of the poly(A) tail and/or decapping of the 5' end allows degradation of them RNA by exonucleases and leads to rapid decay of RNA molecules. In contrast, poly(A) tail elongation prevents enzymatic degradation and increases mRNA stability (Niessing *et al.*, 2002). Moreover, the information available at present show that specific sequences regulate mRNA stability and control translational activation and repression (Huang and Richter, 2004). These sequences is located in the untranslated region present at the 3' end of the mRNA molecule (3' untranslated region-UTR). It has been demonstrated that regulation of maternal mRNA translation is based on changes in the length of the poly(A) tail stretch. Oocyte mRNA displaying short poly(A) tails is translationally inactive. These mRNAs are then activated upon extension of their poly(A) tail at further stages of development. Conversely, many translationally active mRNAs can shorten the poly(A) tail at their 3' end and, consequently, become deactivated (Pocar *et al.*, 2003).

Brevini *et al.* showed that bovine oocytes display a temporal regulation of maternal mRNA polyadenylation during meiotic maturation (Brevini-Gandolfi *et al.*, 1999; Pocar *et al.*, 2001) and the achieved data are

consistent with those available in other species. In their research Brevini and co-workers indicate a possible relationship between the extent of polyadenylation, mRNA stability and the developmental competence during oocyte maturation and demonstrate that an appropriate length of the poly(A) tail at the 3' end must be present, in order to ensure the correct concentration of the encoded protein and adequately support meiosis and early embryo development. Moreover, the role played by oocyte maternal mRNA polyadenylation is not limited to the maturation period, but also contributes to control gene expression and translational activation during the early stages of embryogenesis (Brevini *et al.*, 2002). It is very interesting to remember that a correlation between oocyte developmental competence and polyadenylation levels at the 3'-UTR of specific transcripts has been shown in different species (Yu *et al.*, 2004; Dai *et al.*, 2005). Therefore, it is very appealing to speculate that polyadenylation at the 3'-UTR, mRNA recognition, sorting and localisation may all be aspects involved in the control of both gene expression and, ultimately, oocyte developmental competence. Specific RNA-binding proteins have been described to dock to the sorting transcript and facilitate the anchoring process of the latter at specific sites of the cytoplasm where local translation is promoted. Among the many RNA-binding proteins identified thus far, *Staufen* has been shown to be involved in RNA

localisation in *Drosophila* oocytes (Gavis and Lehmann, 1992; Hachet and Ephrussi, 2004) and *Xenopus laevis* eggs (Yoon and Mowry, 2004).

1.4 *Staufen*-RNA binding protein involvement during oocyte maturation

Proteins specifically interact with mRNAs to regulate their movements and functions. These proteins recognize a diverse array of RNA-binding sites, ranging from single-stranded sequences to perfectly base-paired double-stranded elements (Lunde *et al.*, 2007). The RNA-bound proteins can control localization, translation, or stability of their mRNA targets (Dreyfuss *et al.*, 2002). The double-stranded RNA (dsRNA) binding protein, *Staufen*, participates in all three of these mRNA processes (Roegiers and Jan, 2000) (Kim *et al.*, 2005). The dsRNA-Binding Proteins are a unique family of structurally-related proteins that recognize RNA secondary structure (Saunders and Barber, 2003).

By definition, family members possess at least one dsRNA-binding domain (dsRBD), which consists of an $\alpha\beta\beta\beta\alpha$ fold (St Johnston *et al.*, 1992). A single protein can contain as many as five copies of this domain (Saunders and Barber, 2003; Chang and Ramos, 2005). Multiple dsRBDs within a single protein enable cooperative RNA binding and stabilize the protein-RNA complex (Chang and Ramos, 2005; Parker *et al.*, 2008). Individual dsRBDs are not identical: they exhibit different

RNA binding, and in some cases act as protein-protein interaction domains rather than RNA-binding domains (Broadus *et al.*, 1998; Legendre *et al.*, 2012).

Staufen, a dsRNA-binding protein comprised of five dsRBDs, was initially identified in genetic screens for maternal-effect mutants in *Drosophila*. *Staufen* mutants exhibit multiple embryonic defects including an absence of pole cells, abdomen reduction, and head deformation (Schupbach and Wieschaus, 1986; Lehmann and Nusslein-Volhard, 1991). These defects are caused, at least in part, by mislocalization of mRNAs, such as *oskar* and *bicoid*, in the developing oocyte and embryo (St Johnston *et al.*, 1991; Ferrandon *et al.*, 1994). *Staufen* is also required for efficient localization of *prospero* mRNA during the formation of neuroblasts (Li *et al.*, 1997; Chang and Ramos, 2005). *Staufen* associates with the *bicoid* and *prospero* 3' untranslated regions (UTR) *in vivo*. *Staufen* mediated-localization and binding to *bicoid* mRNA require a highly structured region in the *bicoid* 3'UTR (Ferrandon *et al.*, 1994; Ferrandon *et al.*, 1997; Schuldt *et al.*, 1998). *Staufen* is also required to de-repress mRNAs after localization, implying additional functions in mRNA control. Mammals possess two *Staufen* homologs, STAU1 and STAU2. Together, the results with *Drosophila* and mammalian proteins thus imply functions in localization, translation and decay (Legendre *et al.*, 2012). *Staufen* is a protein that, by binding RNAs, plays a key role in their movement within the cell (see

the schematic graphic representation below) (Figure 2). In particular, it recognizes the mRNA 3'UTR terminal (thanks to their structure) and makes possible the movement along the cytoskeleton facilitating the process of anchoring to the specific sites in the cytoplasm where the translation takes place, i.e. in correspondence of the endoplasmic reticulum (Wickham *et al.*, 1999).

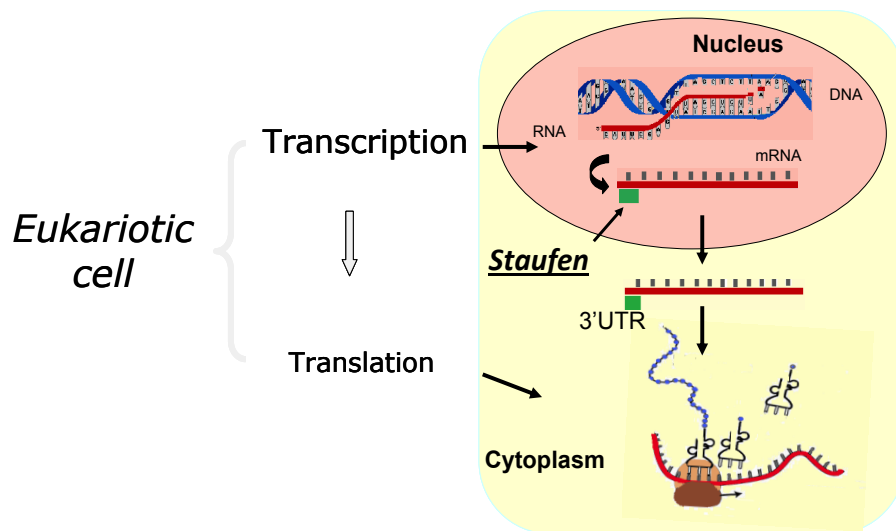


Figure 2: Adapted From Wickham L et al., 1999

The localization of *Staufen* in the cells is mainly in the cytoplasm and to a lesser extent at the nuclear level. It is important to remember that *Staufen* is typically present in highly polarized cells such as epithelial cells, neurons and oocytes. It is also important to point out that the oocyte during maturation has no (or at most negligible) transcriptional activity and therefore the regulation of the processes that take place in

this period is due to the translation of mRNA of maternal origin and / or due to compartmentalization of such mRNA within the cytoplasm. This ensures that there is a correct transcript concentration at the site of translation - where the protein encoded is needed- at the appropriate time. The role of *Staufen* in the localization and transport of mRNA has been studied in *Drosophila* oocytes (Gavis and Lehmann, 1992; Hachet and Ephrussi, 2004), *Xenopus leavis* (Yoon and Mowry, 2004) and in zebrafish (Bateman *et al.*, 2004). Other important studies have been conducted in higher species such as murine (Saunders *et al.*, 2000), bovine (Calder *et al.*, 2008) and porcine (Brevini *et al.*, 2007a). The experiments, carried out in swine, appear to have particular relevance for subsequent studies in humans due to the high structural and functional similarity between the two species in question. In particular, immunolocalization studies carried out on pig oocytes have shown a scattered distribution of *Staufen* during the germinal vesicle (GV) stage, while starting from metaphase I (MI) stage it becomes restricted to the inner region and the oocyte cortex. Once the MII stage is reached granules appears much larger in diameter and spread throughout the cytoplasm except for the area surrounding the spindle. The compartmentalization of *Staufen*, identified in these experiments, recalls the reorganization of the endoplasmic reticulum described in oocytes from different species (Otsuki *et al.*, 2004; Ebner *et al.*, 2008).

These considerations, therefore, allow us to hypothesize a possible association between *Staufen* and the endoplasmic reticulum in human oocyte. In particular, this protein would facilitate a correct localization of maternal mRNA to the specific sites of translation corresponding to specific portions of the reticulum, following a precise a temporal/space adjustment intimately connected with the process of the female gamete maturation.

1.5 Role of *Calreticulin* during oocyte maturation

Calreticulin (CALR) is a multifunctional protein that acts as a major Ca^{2+} -binding (storage) protein in the lumen of the endoplasmic reticulum (ER) (Gelebart *et al.*, 2005) (see Figure 3). It is also found in the nucleus, suggesting that it may have a role in transcription regulation. CALR binds to the synthetic peptide KLGFFKR, which is almost identical to an amino acid sequence in the DNA-binding domain of the superfamily of nuclear receptors, it is highly conserved among species, and it is located in the endoplasmic and sarcoplasmic reticulum where it may bind calcium. The amino terminus of CALR interacts with the DNA-binding domain of the glucocorticoid receptor and prevents the receptor from binding to its specific glucocorticoid response element. *Calreticulin* can inhibit the binding of androgen receptor to its hormone-responsive DNA element and can inhibit androgen receptor and retinoic

acid receptor transcriptional activities in vivo, as well as retinoic acid-induced neuronal differentiation. Thus, CALR can act as an important modulator of the regulation of gene transcription by nuclear hormone receptors.

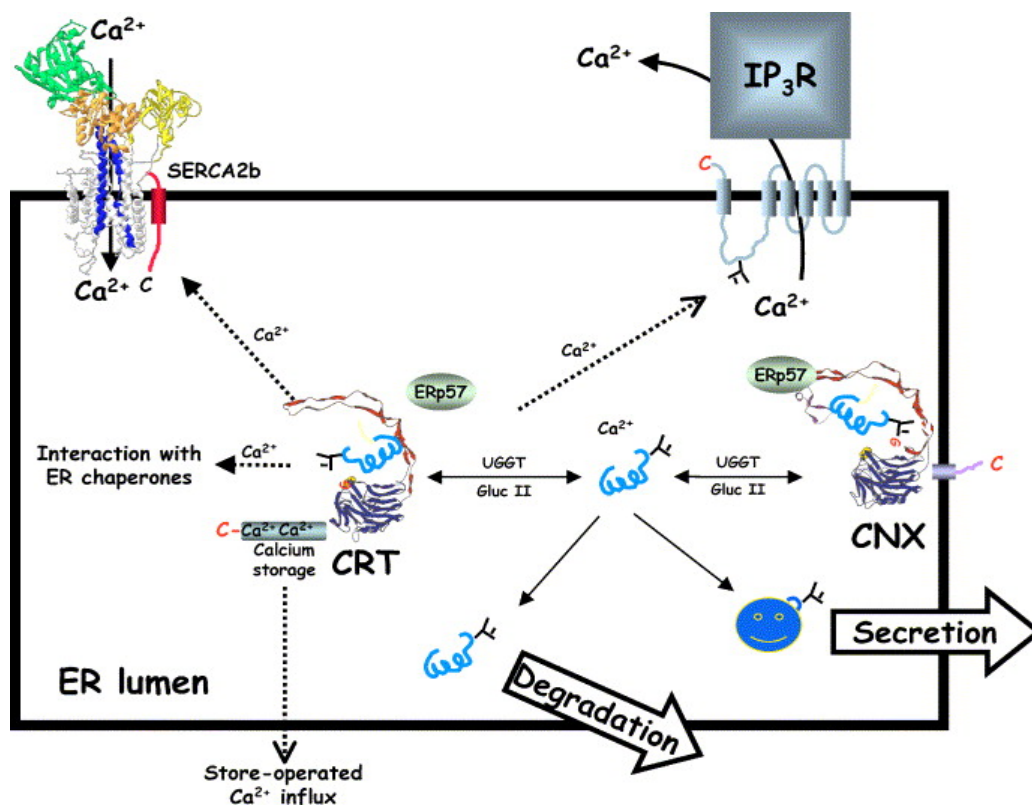


Figure 3: From Gelebart (Gelebart *et al.*, 2005) *Calreticulin* in the lumen of the ER. This diagram shows a model of events occurring in the ER lumen. From details see text and reference. CRT: *Calreticulin*; CNX: calnexin; PDI: protein disulfide isomerase; Gluc II: glucosidase II; UDP-Gluc transferase: UDP-glucose:glycoprotein glucosyltransferase; UP: unfolded glycoprotein; G: glucose residue.

CALR is a protein of 46 Kd localized in the lumen of the endoplasmic reticulum and consists of three distinct domains, of which those N-terminal and central have a chaperonic activity, whereas the C-terminal binds ion Ca^{2+} with low affinity and high capacity. It is estimated that one mole of CALR can bind 20-25 moles of Ca^{2+} (Nakamura *et al.*, 2001). The function of the chaperon CALR is carried out in association with the calnexin so that the glycoproteins of recent synthesis assume a correct conformation before being released from the organelle - the ER - to be transferred to the respective final destinations (Michalak *et al.*, 2009). The function of binding of Ca^{2+} of the CALR is crucial to the role played by this ion in important cellular processes, such as adjusting the cytoskeleton, modulate gene expression and protein synthesis and as a signal of cellular stress (Wang *et al.*, 2012). In oocytes, the Ca^{2+} plays the well-known role of regulating fertilization being involved in the maturation process and allowing completion of meiosis (Zhang *et al.*, 2010). The RE is the main organelle for storage of Ca^{2+} ions, ensuring intracellular homeostasis. The release of Ca^{2+} from the ER is regulated by the inositol triphosphate receptor (InsP3R) and ryanodine, while the accumulation is mediated by the pump IF / ER Ca^{2+} ATPase (SERCA) (Coe and Michalak, 2009). CALR interacts with all of these Ca^{2+} binding molecules regulating their functions. For example, it is believed that the high capacity of the CRT to bind Ca^{2+} could inhibit an excessive release

of Ca^{2+} from the ER – due to the InsP3 receptor stimulation – avoiding the endoplasmic reserve depletion and simultaneously preventing that a massive output of Ca^{2+} results toxic for the process taking place in cytosol (John *et al.*, 1998). In mammalian oocytes, CALR seems to play an important role in calcium homeostasis during oogenesis and development. In pig oocytes CALR mRNA is present at all stages of maturation (Zhang *et al.*, 2010). It has been noted that the perturbation of Ca^{2+} homeostasis obtained by inhibition of ATPase interfere with meiotic maturation, the polyadenylation of messengers of important regulators of oocyte function (such as GDF9 and C-MOS) and the early stages of embryonic development (Zhang *et al.*, 2010). This demonstrates the importance of Ca^{2+} for oocyte regulation and the critical role of Ca^{2+} reserves constituted by RE, organelle of which the CALR is an important element. Studies in epithelial cells have also been shown that during the cell cycle, distribution of *Calreticulin* follow a specific pattern. By immunofluorescent and electron microscopy, it has been observed that, in interphase, staining for CALR distinguishes a reticular network comprising the endoplasmic reticulum and the nuclear envelope (Ioshii *et al.*, 1995). This staining fully coincides with one arising from the use of DiOC6, a dye specific for RE, which showed that the RE is the elective compartment for CALR localization (and accumulation of Ca^{2+}). At different stages of the cell cycle, the localization of the CALR significantly changes, i.e. from the pro-

metaphase and subsequently throughout the mitotic phase. In particular, in mitotic cells the CALR, as well as being widely distributed in the cytoplasm, focuses specifically and abundantly in tubulo-reticular structures surrounding the mitotic spindle. The different distribution of the CALR during the mitotic cycle suggests that this protein may interact with different cellular structures and therefore play different roles at different stages of cell life (Ioshii *et al.*, 1995).

2. AIM OF STUDY

Preovulatory maturation represents the final act of oogenesis during which not only meiotic maturation is resumed and finalized, but also the cytoplasmic compartment undergoes major modifications that are crucial for the acquisition of fertilization and developmental ability. Virtually all organelles change localization and distribution, becoming more competent in supporting the sperm-elicited activation mechanisms and early cleavage events. During maturation, informational molecules are also subject to regulatory influences. In fact, as an effect of GV breakdown, RNA molecules are rather abruptly exposed to cytoplasmic factors that, through a plethora of mechanisms, may determine their stabilization, degradation or translation. In particular, RNA relocalization and confinement are regulatory modalities exquisitely important to a very large and highly polarized cell, such as the fully-grown oocyte, in order to achieve exclusive and maximal concentration of a newly translated protein within a specific cytoplasmic domain.

In this scenario, *Staufen* RNA Binding Protein may play an important role in the light of its function of vector of mRNAs from the nucleus to the sites of translation. Therefore, we hypothesized that *Staufen* is expressed during oocyte maturation and that the localization of *Staufen* protein changes during the meiotic transition from GV to MII. We also

postulated that the pattern of *Staufen* distribution reflects its physical interaction with the endoplasmic reticulum, an organelle directly involved in the process of mRNA translation whose relocalization is central to the process of oocyte maturation.

To test the above hypotheses, we assessed the expression of *Staufen* and *Calreticulin* (CALR), the latter adopted as a marker of the endoplasmic reticulum, in human oocytes at different stages of maturation: GV, MI and MII.

The oocytes were obtained from supernumerary material of assisted reproductive cycles and were subjected to polymerase chain reaction in order to investigate the expression of *Staufen*, CALR and related RNAs. The corresponding protein products were identified by immunofluorescence and confocal laser scanning microscopy.

Our findings indicate that *Staufen* and CALR are constantly expressed and selectively localized during oocyte maturation. The different pattern of *Staufen* distribution at the GV, MI and MII stages implicates that localization of protein translation is one of the levels at which gene expression is regulated in the maturing oocyte. On the other hand, localization of CALR at the MII stage reveals novel aspects of physical interaction with the MII spindle and suggests a previously unrecognised role of the endoplasmic reticulum in support of cytoskeletal function in the human oocyte.

3. MATERIALS AND METHODS:

3.1 Oocyte retrieval from patients undergoing Assisted Reproduction Treatment

Oocytes were collected from consenting patients undergoing in-vitro fertilization (IVF) treatments (Figure 4) for different causes of infertility but, for this study, from “normo-ovulatory” patients. Spare eggs, not utilized for the reproductive cycle, were donated for research purposes and fixed or cryopreserved utilizing a slow cooling protocol. Ovarian stimulation was conducted according to a long standard stimulation protocol (Figure 5) using gonadotrophin-releasing hormone agonist (GnRH)(Enantone[®]; Takeda, Osaka, Japan) and recombinant Follicle Stimulating Hormone (r-FSH) (Puregon[®]; MSD or Gonal F[®], MerckSerono, Geneve, Switzerland). Ovulation was triggered with (human Chorionic Gonadotrophin) hCG (Ovitrelle[®] or Gonasi, [®], MerckSerono, Geneve, Switzerland). Oocyte retrieval was performed transvaginally, under ultrasound guidance, 36 hours after hCG injection

using a CCD aspiration needle 17 Gauges (CCD, Paris, France) (Figure 6).

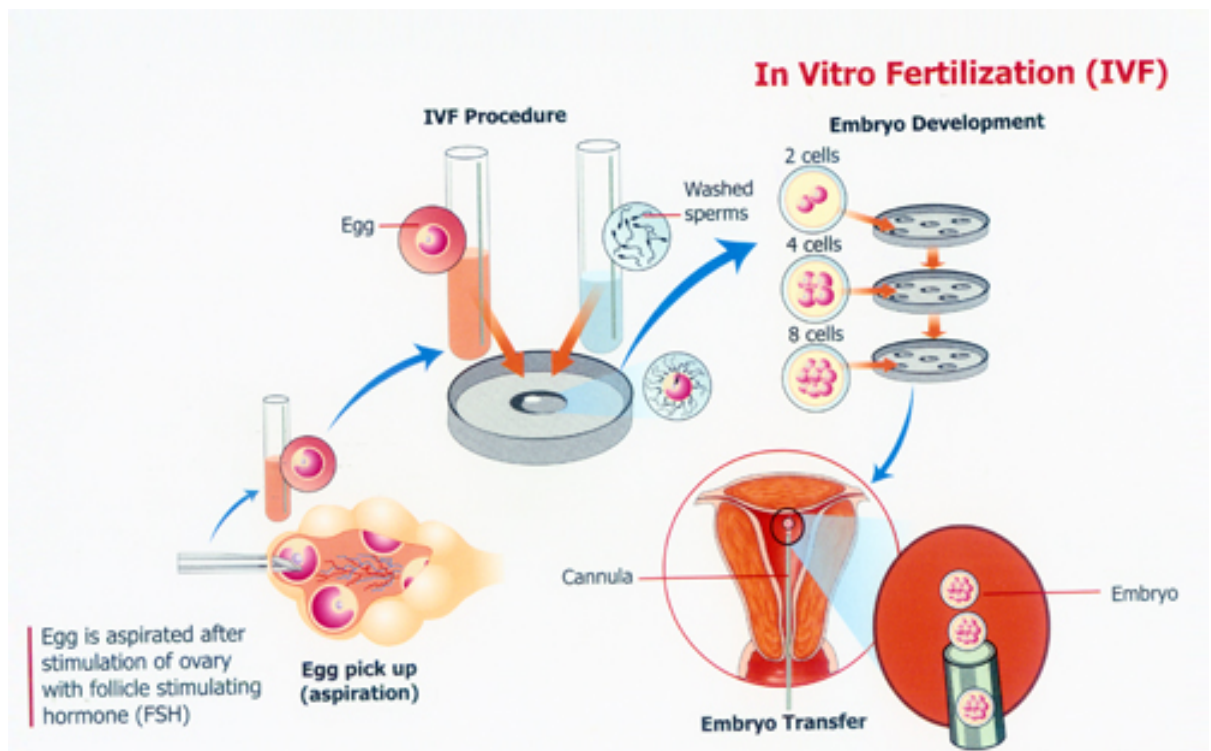


Figure 4: Schematic representation of the laboratory competence during the In Vitro Fertilization and embryo transfer procedure IVF-ET.

From siddhilifeivf.com

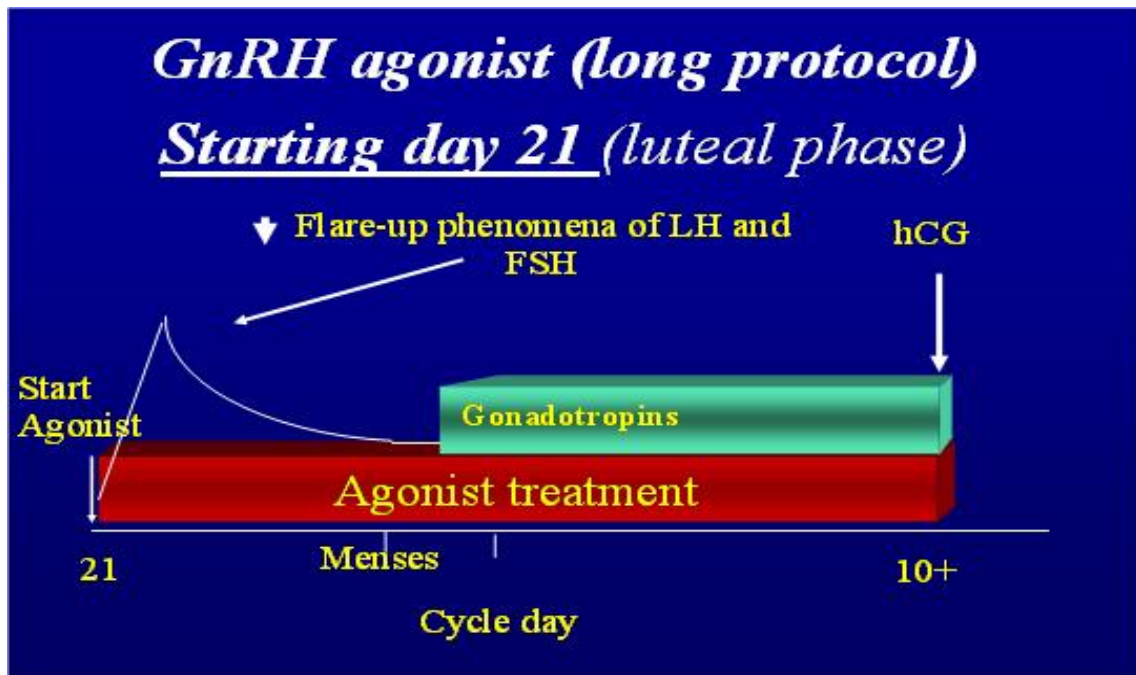


Figure 5: Conventional Long Stimulation Protocol (from <http://www.ivf-worldwide.com/Education/stimulation-protocols.html>)

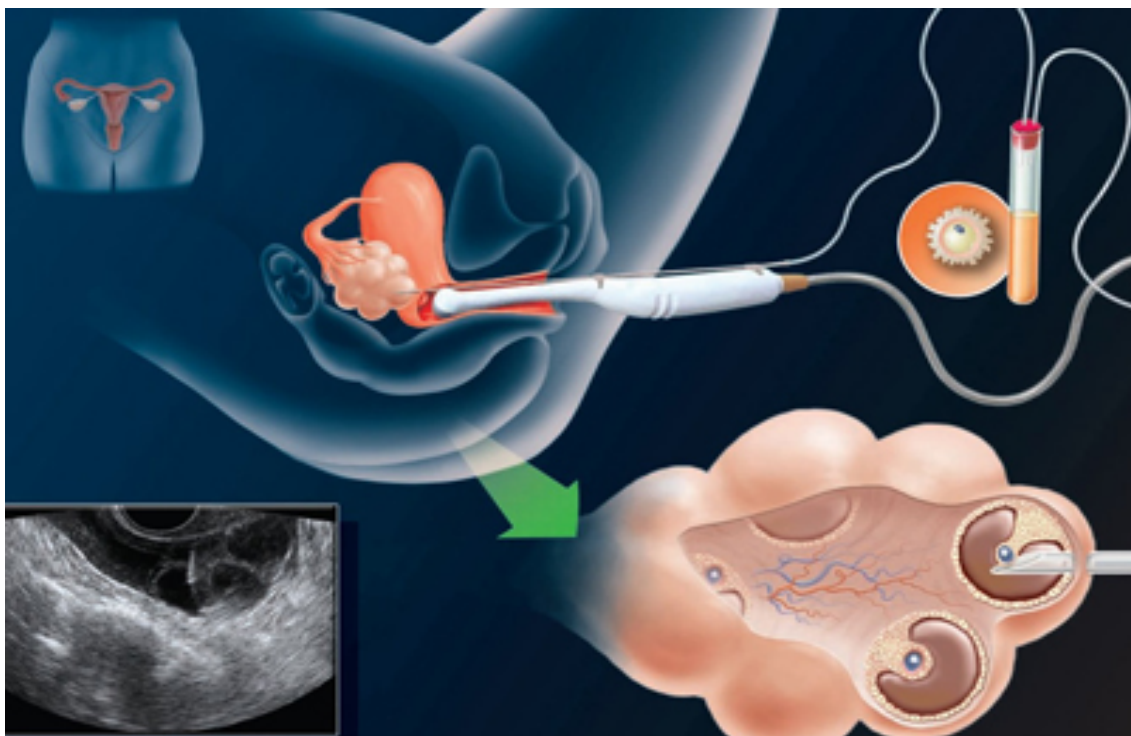


Figure 6: Egg collection procedure, well known as oocyte pick up (From MerckSerono, Geneva, Switzerland)

3.1.1 Assessment of nuclear status

Cumulus and corona radiata cells were immediately removed after retrieval by a short exposure to HEPES-buffered medium (Quinn's Advantage Hepes Medium, Sage IVF Inc, Trumbull, CT, USA) containing 20 IU/ml hyaluronidase (Sage IVF Inc, Trumbull, CT, USA) and gentle aspiration in and out of a hand drawn pipette (Flexi-Pet™, Cook, Australia) and mechanically cleaned from the remaining surrounding cumulus cells by aspiration using a denuding pipette with a 170–130 µl diameter (Denuding Flexi-Pet™; Cook, Australia). The denuded oocytes were then assessed with respect to their meiotic maturation status (GV,MI or MII) (Figure 7).

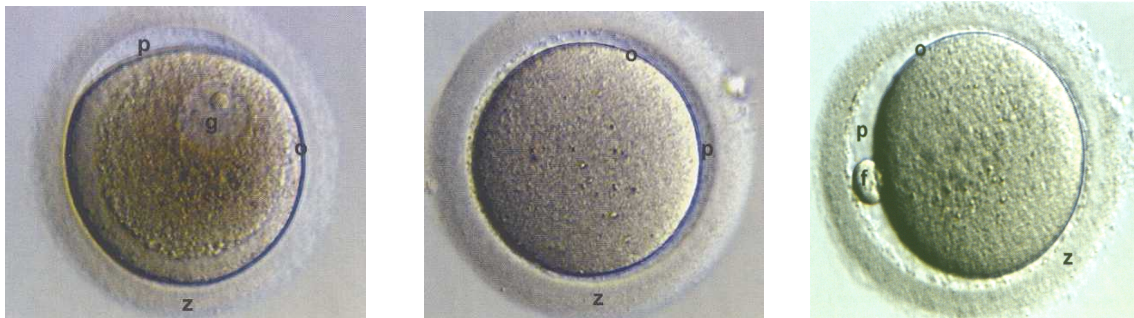


Figure 7: Human oocyte at the GV (left), MI (middle) and MII (right) stage after cumulus-corona cells removal. "p" indicates the perivitelline space, "o" the oolemma and "z" the zona pellucida. "g" and "f" point out the germinal vesicle and the first polar body respectively

3.1.2 Oocyte cryopreservation

The oocytes were cryopreserved using a slow cooling method with two alternative protocols differing in sucrose concentration (De Santis *et al.*, 2007b). All cryopreservation solutions were prepared using Dulbecco's phosphate-buffered solution (PBS) (Gibco, Life Technologies Ltd, Paisley, Scotland) and a Plasma Protein Supplement (PPS) (BAXTER AG, Vienna, Austria). The two freezing solutions were constituted as follow: (a) 1.5 mol/l PrOH + 20% PPS in PBS and (b) 1.5 mol/l PrOH + 0.1 (or 0.2 or 0.3) mol/l sucrose + 20% PPS in PBS. The thawing solution were prepared as follow: (1) 1.0 mol/l PrOH + 0.2 (or 0.3) mol/l sucrose + 20% PPS; (2) 0.5 mol/l PrOH + 0.2 (or 0.3) mol/l sucrose + 20% PPS; (3) 0.2 (or 0.3) mol/l sucrose + 20% PPS, (4) PBS+20% PPS.

Within four hours after retrieval oocytes were incubated, at room temperature, in a PBS solution containing 1.5 mol/l PrOH for 10 min and then in a solution of 1.5 mol/l PrOH + 0.1 mol/l sucrose or 1.5 mol/l PrOH + 0.3 mol/l sucrose, and subsequently loaded into plastic straws (paillettes souple 0.1 ml Cryo Bio System, CBS, France). A maximum of 3 oocytes per straw was loaded (Figure 8). Each straw was sealed using a CBS High security sealer (CBS, France) and was expressly identified by permanent labelling. The straws were then allocated in a Planer CRYO 360- 1.7 (Sapio Life, Monza, Italy) programmable freezing device (Figure 9). A slow cooling protocol was

used and manual seeding was performed at -8°C . Thawing was conducted at room temperature, with a four step procedure: 5 min in 1 mol/l PrOH + 0.2 mol/l sucrose or 0.3 , 5 min in 0.5 mol/l PrOH + 0.2 mol/l sucrose or 0.3 , 10 min in 0.2 mol/l sucrose or 0.3 , 10 min in PBS. Both freezing and thawing solutions were supplemented with plasma protein solution (20%V/V). Oocytes were then replaced in culture medium at 37°C for at least one hour until further evaluation of oocytes degeneration (De Santis *et al.*, 2007a; De Santis *et al.*, 2007c) (Figure 10)

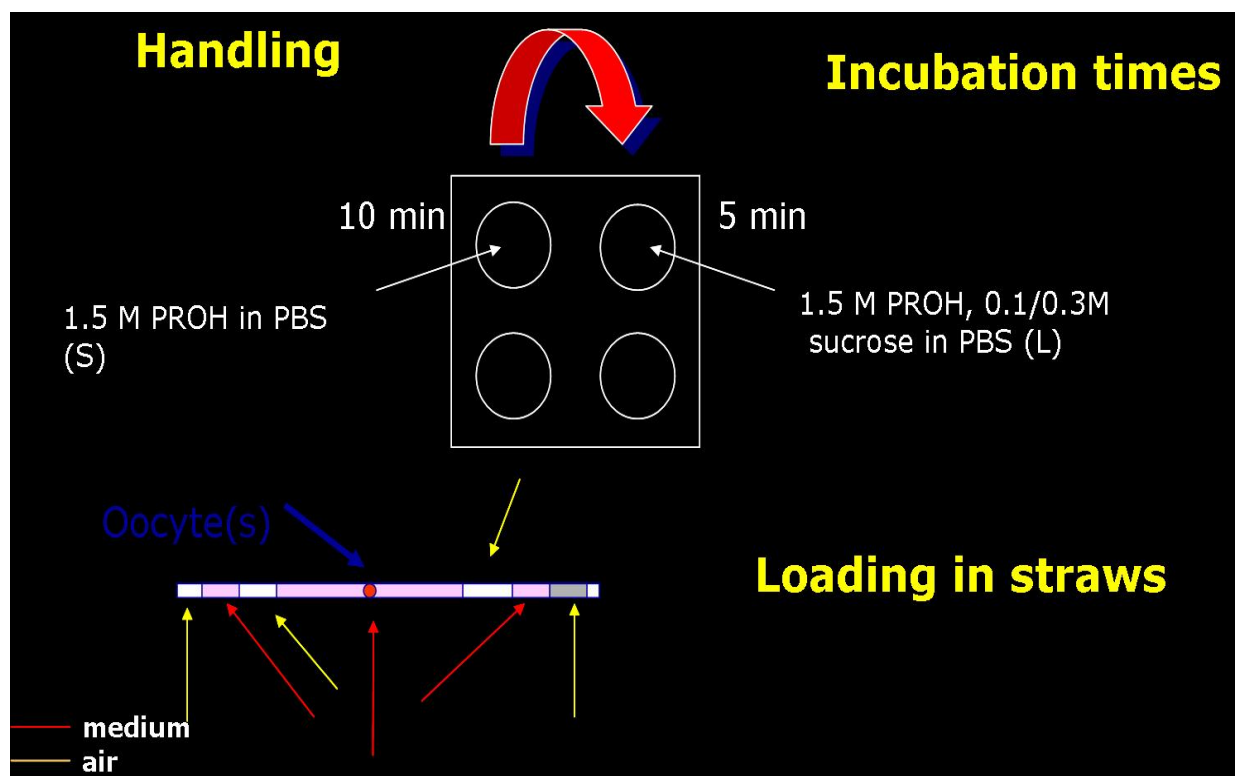


Figure 8: equilibration procedures and loading of oocytes before slow freezing (De Santis *et al.*, 2007a)



Figure 9: Planer CRYO 360- 1.7 (Sapio Life, Monza, Italy)

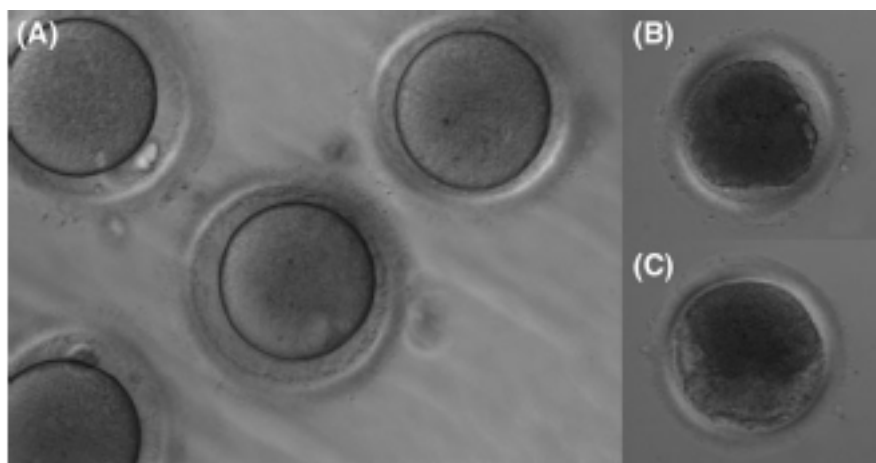


Figure 10: Representative micrographs of oocytes considered viable (A) or degenerate (B and C) after thawing and incubation for at least 3 h (De Santis *et al.*, 2007c)

3.2 Molecular approach for quantitative analysis of *Staufen* and *Calreticulin* messengers

3.2.1 RNA extraction

Oocytes were pooled in groups of five for poly(A)⁺ RNA extraction which was carried out using the Dynabeads mRNA DIRECT Micro-kit according to the manufacturer's instructions. More in details, oocyte pools were lysed for 10 min at room temperature in 100 µl lysis buffer (100 mM Tris-HCl pH 7.5, 500 mM LiCl, 10 mM EDTA pH 8.0, 1% (w/v) LiDS, and 5 mM dithiothreitol (DTT)). After lysis, 20 µl of pre-washed Dynabeads oligo(dT)₂₅ were pipetted into the tube and binding of poly(A)⁺ RNAs to oligo(dT) was allowed for 5 min at room temperature. The beads were then separated with a magnetic separator, washed twice with 100 µl washing buffer A (10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA, and 0.1% (w/v) LiDS) and thrice with 100 µl washing buffer B (10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA). Poly(A)⁺ RNAs were then eluted from the beads by incubating in 8.5 µl DEPC-treated sterile water at 65°C for 5 min. Aliquots were immediately used for RT.

3.2.2 RNA reverse transcription

RNA was reverse transcribed into cDNA in a total volume of 20 μ l reaction mixture containing 8.5 μ l sterile water, 1 μ l of 10 mM dNTP mix, and 1 μ l oligo(dT)₁₂₋₁₈ (500 ng/ μ l). RNA was denatured at 65 °C for 5 min, and then 4 μ l of 5x first-strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 15 mM MgCl₂), 1.5 μ l of 50 mM MgCl₂, 2 μ l of 0.1 M dithiothreitol, and 1 μ l of RNaseOUT recombinant RNase inhibitor (40 U/ μ l) were added. RT was performed with 200 U Superscript II reverse transcriptase for 1 h at 42 °C. Enzymes were inactivated at 70 °C for 15 min.

3.2.3 Semi-quantitative *Polymerase Chain Reaction (PCR)*

An aliquot of each RT product was subjected to gene-specific PCR in an automated thermal cycler (iCycler, Bio-Rad, Italy). PCR was performed with cDNA equivalents corresponding to 0.25 oocyte. The reaction mix consisted of 0.8 μ l of 50 mM MgCl₂, 0.3 μ l Taq DNA polymerase (5 U/ μ l), 2 μ l of 10x PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 1 μ l of 10 mM dNTPs, 1 μ l (25 pmol) of sequence-specific primer, and sterile water up to 20 μ l. The sequence of primers used were: *Staufen*

FOR 5'- cacctccgtgtttggtcttt-3' and REV 5'- ggtcacgctgagtaggaagc -3',
28S FOR 5'- ggtagttccaatgccaagga -3' and REV 5'-
aagatccgtccaatgaccag -3'. The optimal cycle number at which the
transcript was amplified exponentially was established running a linear
cycle series and the number of PCR cycles was kept within this range.
RT-PCR products were subjected to electrophoresis on 2% agarose gel
in 1x TAE buffer (40 mM Tris-acetate and 1 mM EDTA) containing 0.5
µg/ml ethidium bromide. After electrophoresis at 80 V for 45 min, the
fragments were visualized on a 312 nm U.V. transilluminator. The image
of each gel was recorded using a Kodak digital camera. The intensity of
each band was assessed by densitometric analysis, performed with the
Quantity One® software (Bio-Rad, Italy). The quantification procedure
based on direct digitalization of the PCR product after separation on
agarose gel provides a well-established and sensitive method to detect
even small differences in amounts of mRNA from different biological
samples (Grover *et al.*, 2001). The relative amount of the mRNA of
interest was calculated by dividing the intensity of the band for each
gene of interest by the intensity of the 28S band, as described
previously (Cillo *et al.*, 2007). To account for gel to gel variations, each
band was normalized against the 350 bp band of a 50 bp ladder marker
(Stenman *et al.*, 1999) .

3.2.4 Oocytes gene expression

The primers used in these experiments were designed based on human genome sequence available in database bank using the oligo program Primer3 Input. PCR were carried out using the conditions specific of each pair of primers (Table 1).

GENE	Accession Number	PRIMERS	Tm (°C)	Amplification product
<i>Staufen</i> (STAU)	NM_017454.2	FOR 5'- cacctccgtgtttggtcttt -3' REV 5'- ggtcacgctgagtaggaagc -3'	60	183bp
<i>Calreticulin</i> (CALR)	NM_004343.3	FOR 5'- tctcagttccggcaagttct -3' REV 5'- tctgagtctccgtgcatgtc -3'	60	231bp
Homo sapiens heat shock 70kDa protein 5 (HSPA5)	NM_005347.4	FOR 5'- tagcgtatggctgctgctgc -3' REV 5'- tttgtcaggggtctttcacc -3'	60	241bp
Nestin (NES)	NM_006617.1	FOR 5'- aacagcgacggaggtctcta -3' REV 5'- ttctctgtcccgcagactt -3'	60	220bp
Homo sapiens ribosomal protein S28 (RPS28)	NM_001031.4	FOR 5'- tccatcatccgcaatgtaaa -3' REV 5'- tgtgacagaccattcccatc -3'	60	157bp

Table 1: List of primers used for PCR detailing accession number, primer sequence, annealing temperature and expected amplification product size.

Expression of 28S was used as an internal control of the sample quality. HSPA5 (BIP) was examined to validate and reinforce CALR expression, Nestin was amplified as negative control. To confirm the identity of the RT-PCR fragments, amplification products from each primer pair were separated by gel electrophoresis in 2% agarose gels, purified using Spin-X centrifuge tube filters (Corning, Italy), sequenced (SEQLAB, Gottingen, Germany) and aligned using Clustal W 1.82 (EMBL-EBI service).

3.3 Confocal Microscopy

Stained oocytes were examined at the CIMA (Centro Interdipartimentale di Microscopia Avanzata) microscopy centre of this University using a TCS-NT laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany) equipped with Ag/Kr and He/Ne lasers. Blocking filters used were band path, 530 ± 30 (*Staufen* and or *Calreticulin*) and long path, 450 (DNA). *Staufen* and *Calreticulin* distribution was assessed through equatorial optical sections of 6.7 mm thickness. Laser intensity was 1.5 mV. Objective (103 and 403 Leica Floutar; Leica Microsystems, Heidelberg, Germany), pinhole (1 Airy unit), filters, offset, gain, and Photon Multiply Tube settings were kept constant throughout the experiments. *Staufen* and *Calreticulin*

distribution was assessed by sequential scanning of at least 40 μm of sample with 2.6 μm step size and controlled oversampling. Depending on different availability, confocal analysis was conducted using the "Alembic" microscopy facility of Vita-Salute San Raffaele University equipped with a Leica TCS SP2 Laser Scanning Confocal Microscope using the same settings.

3.3.1 *Staufen immunolocalization*

Immuno-cytochemical analysis was initially carried out on 10 oocytes per each stage of maturation (GV, MI and MII). Each oocyte was fixed in 4% paraformaldehyde in PBS for 20/30 min at 4°C and then permeabilized with 0.5% Triton X-100 in PBS overnight at 37°C. Oocytes were washed in PBS and incubated in a 0.1 M NH_4Cl solution for 10 min at room temperature. After blocking in 5% bovine albumin serum (BSA) in PBS, they were incubated with an anti-human *Staufen* rabbit antibody (1:200, Abcam, UK, Prodotti Gianni, Italy) for 1 h at room temperature. Secondary detection was carried out with Alexa Fluor 488 antibody. DNA was stained with 4',6'-diamidino-2-phenylidole (DAPI; 0.2 $\mu\text{g}/\text{ml}$). At the end of the procedure, samples were mounted on glass slides in an anti-fading mounting medium (Pro-Long, Molecular

Probes, Italy). They were then stored below 0°C until confocal microscopy was performed.

When immediate staining was not possible oocytes were fixed in 4% paraformaldehyde in PBS for 30 min at 4°C and then washed three times in PBS/PVA and stored at 4°C. In the last period of the study for the relative difficulties in recruitment MI stage oocytes, the analysis on this meiotic stage was discontinued and maintained only on GV and MII. Aiming to increase the material available for the project also MII cryopreserved oocytes were thawed (as described above) and the survived cells were fixed and stained with the same protocol illustrated for fresh material.

3.3.2 *Staufen* distribution analysis

Confocal images were processed using MacBiophotonics ImageJ dedicated software (<http://www.macbiophotonics.ca/imagej/>). The area of *Staufen* spots, present in each captured image was measured. *Staufen* spot size was assessed in oocytes at different developmental stages (GV, MI and MII). Mean area of each stage was then calculated and spots were classified as \geq or $<$ than mean value.

3.3.3 *Calreticulin immunolocalization*

Immuno-cytochemical analysis was initially carried out on 15 oocytes per each stage of maturation (GV and MII). As for the last part of the study on *Staufen*, MI were excluded in relation to difficulties in recruitment human oocyte at this stage of maturation following controlled ovarian hyperstimulation and the paucity of biological material available for research following the modification of Italian IVF law. Each oocyte was fixed in 4% paraformaldehyde in PBS for 20/30 min at 4°C and then permeabilized with 0.5% Triton X-100 in PBS overnight at 37°C. Oocytes were washed in PBS and incubated in a 0.1 M NH₄Cl solution for 10 min at room temperature. After blocking in 5% bovine albumin serum (BSA) in PBS, they were incubated with an anti-human *Calreticulin* rabbit antibody (1:200, Abcam, UK, Prodotti Gianni, Italy) for 1 h at room temperature. Secondary detection was carried out with Alexa Fluor 488 antibody. DNA was stained with Hoechst 33258 (Molecular Probes). At the end of the procedure, samples were mounted on glass slides in an anti-fading mounting medium (Pro-Long, Molecular Probes, Italy). They were then stored below 0°C until confocal microscopy was performed.

When immediate staining was not possible oocytes were fixed in 4% paraformaldehyde in PBS for 30 min at 4°C and then washed three times in PBS/PVA and stored at 4°C. Aiming to increase the material

available for the project also MII cryopreserved oocytes were thawed (as described above) and the survived cells were fixed and stained with the same protocol illustrated for fresh material.

3.3.4 *Calreticulin distribution analysis*

For *Calreticulin* staining was evaluated as the presence/absence of green fluorescence localization in GV or MII oocytes and, as for *Staufen*, images were processed using MacBiophotonics ImageJ dedicated software. Some images were analyzed graphically as shown in the results chapter.

3.3.5 *Actin Staining*

The staining for filamentous actin (well known as f-actin) was carried out simultaneously with that of the other proteins (*Staufen* or *Calreticulin*). In particular, during the incubation with the secondary antibody, samples were stained with rhodamine-phalloidin (1:200; Molecular Probes) for 1 hour at 37°C. After washing, oocytes were mounted and then images were collected.

3.3.6 Chromosomes Staining

The staining for chromatin detection is a part of the entire procedure for *Staufen* or *Calreticulin* staining. Most of the acquisition for *Staufen* localization utilized DAPI as described in the section dedicated to *Staufen* staining procedure, however for simultaneous *Calreticulin* staining we preferred to utilize Hoechst 33258 giving its fluorescence a sharper image definition. In detail, during the last step of incubation with the secondary antibody 1 µg/ml Hoechst 33258 (Molecular Probes) was also added. Finally, after three steps of washing (10 minutes each), oocytes were mounted using 50% glycerol/PBS solution containing 25 mg/ml sodium azide and 1 µg/ml Hoechst 33258.

4.RESULTS

4.1.1 *Staufen* expression in human maturing oocytes

Data obtained indicate that *Staufen* is expressed during all stages of oocyte maturation suggesting a possible physiological role of this protein in humans, likewise to what described in other species.

Our experiments aimed at confirming the results obtained by immunocytochemical approach through the use of molecular techniques. In this set of experiments, pools of 5 oocytes in GV, MI and MII stage respectively were subjected to semi-quantitative polymerase chain reaction (RT-PCR) screening for the evaluation of *Staufen* expression pattern.

The expression levels of *Staufen*, acquired after molecular analysis, were consistent with the data obtained via immunocytochemical evaluation (see section 3.2.1) and showed a constant expression of the transcript during the maturation process (see chart below) (Figure 11)

Staufen expression

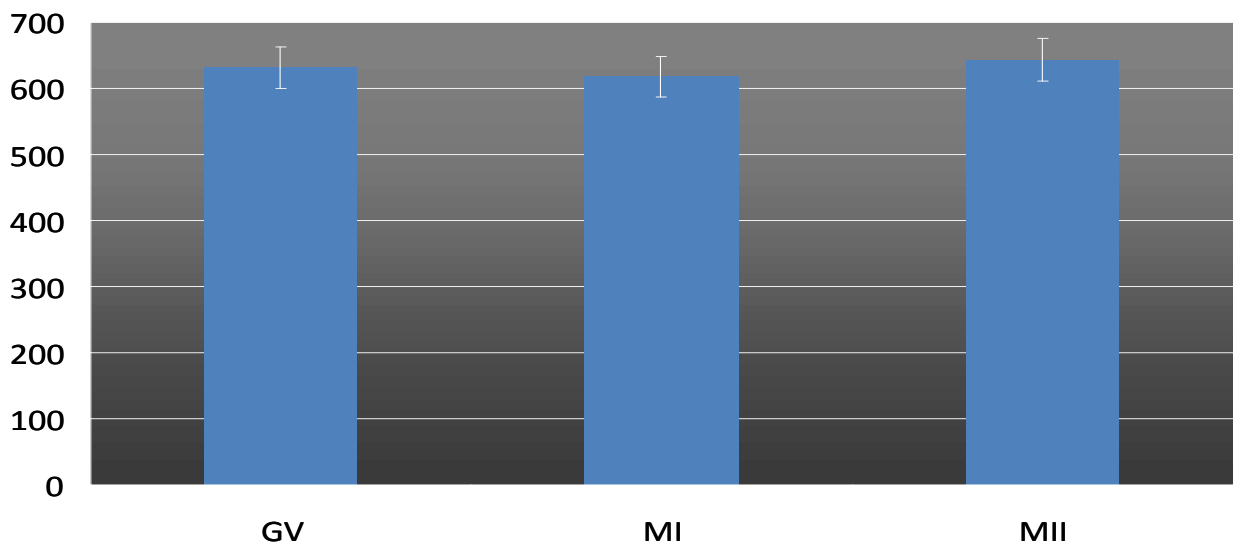


Figure 11: Graphic representation of *Staufen* expression levels in human oocytes during the three maturational stages of Germinal Vesicle, Metaphase I and Metaphase II

4.1.2 Expression of *Calreticulin* in Metaphase II human oocytes

Similarly to what was done for the analysis of *Staufen* expression, molecular studies have been carried out in order to evaluate the expression of CALR. To this purpose specific primers were designed. Unfortunately, in this set of experiments - due to new rules in the IVF laboratory management - only MII stage oocytes were accessible for analysis as there was no availability of oocytes donated for research at the GV and MI stage. For a more comprehensive analysis, CALR

expression was linked again to *Staufen* and to HSPA5. The protein encoded by the latter gene is a member of the heat shock protein 70 (HSP70) family. It is localized in the lumen of the endoplasmic reticulum (ER), and is involved in the folding and assembly of proteins in the ER. As this protein interacts with many ER proteins, it may play a key role in monitoring protein transport through the cell.

28S (Structural RNA of the larger unit of the ribosome) was used with the function of "house keeping" gene and therefore as a positive control in opposition to Nestin (whose expression is peculiar of nerve cells) used as negative control. The results are summarized in the table below and indicate the presence of both CALR and HSPA5 proteins in human MII oocytes, implying that the mechanisms involved in sorting, folding and assembly of proteins to the ER are active in the matured human gamete. (Table 2)

GENE	MII OOCYTES
<i>Staufen</i>	+
<i>Calreticulin</i>	+
HSPA5 (BIP)	+
Nestin	-
28S	+

Table 2: CALR and HSPA5 expression in human MII oocytes evaluated together with *Staufen*, 28S and Nestin

4.2.1 *Staufen* localization in human maturing oocytes

Immunocytochemical approach has been performed to characterize the localization of *Staufen* in human oocytes assessed in three different stages of maturation: germinal vesicle (GV), metaphase I (MI) and metaphase II (MII).

The results showed that *Staufen* is expressed in human oocytes at different stages of maturation. The collected data also indicate interesting peculiarity with respect to the distribution of *Staufen*, within the cytoplasm, with a temporal-space adjustment that appears to be correlated to the process of maturation.

The immunolocalization of *Staufen* was conducted on 30 human oocytes at different stages of maturation, classified as germinal vesicle (10-GV), metaphase I (10-MI) and metaphase II (10-MII) stage of maturation (Figure 12).

All human oocytes analyzed were positive for *Staufen*, thus demonstrating that in the human the expression of this protein is present during the different stages of maturation.

Once having acquired through analysis by confocal microscopy 15 sections for each oocyte, it was possible to detect a characteristic

distribution of *Staufen* for the different stages of maturation. The images below are just representative of the different distribution of *Staufen* depending on the different stage of maturation of human oocytes (Figure 12).

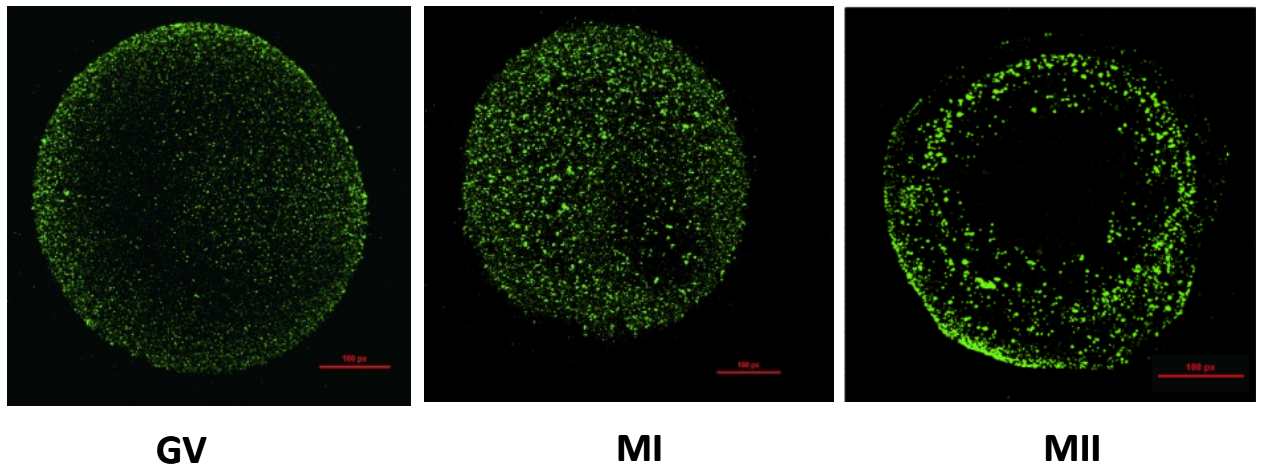


Figure 12: *Staufen* (RNA-binding protein) localization in the human maturing oocyte

At the GV stage the protein shows a dispersed localization throughout the cytoplasm. At the MI *Staufen* begins to compartmentalize towards the cortical area of the oocyte organizing into granules of larger dimensions.

The polarization to the cortex is even more evident in MII stage where it is possible to observe a discrete number of granules that predominantly occupy the peripheral part of the ooplasm.

The images acquired by confocal microscopy were analyzed in order to:

- a) quantify the total area of the oocyte;
- b) calculate the total number of positive granules for *Staufen*;
- c) measuring the mean area of the granules at different stages of maturation.

These parameters were evaluated and led to the results listed below. By setting a threshold value of the dot size by the average area of the spot relative to *Staufen*, specific to each stage of maturation, it was possible to make an assessment expressed in percentage of the area of the spot.

The values obtained are shown in the "pie charts" (Figure 13)

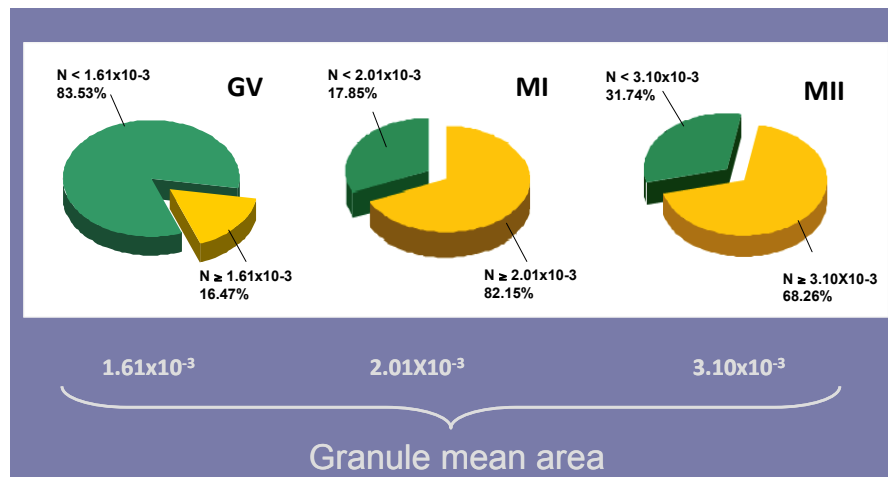


Figure 13: "pie chart" graphic showing the change - in terms of number and diameter - of the fluorescent spots stained for *Staufen*

4.2.2 *Calreticulin* localization during transition from Germinal Vesicle to Metaphase II stage in human oocytes

Human oocytes at different stages of development were isolated from adjacent cumulus cells, fixed and labelled to highlight actin, DNA and more specifically CALR, and then observed by confocal microscopy (Figure 14)

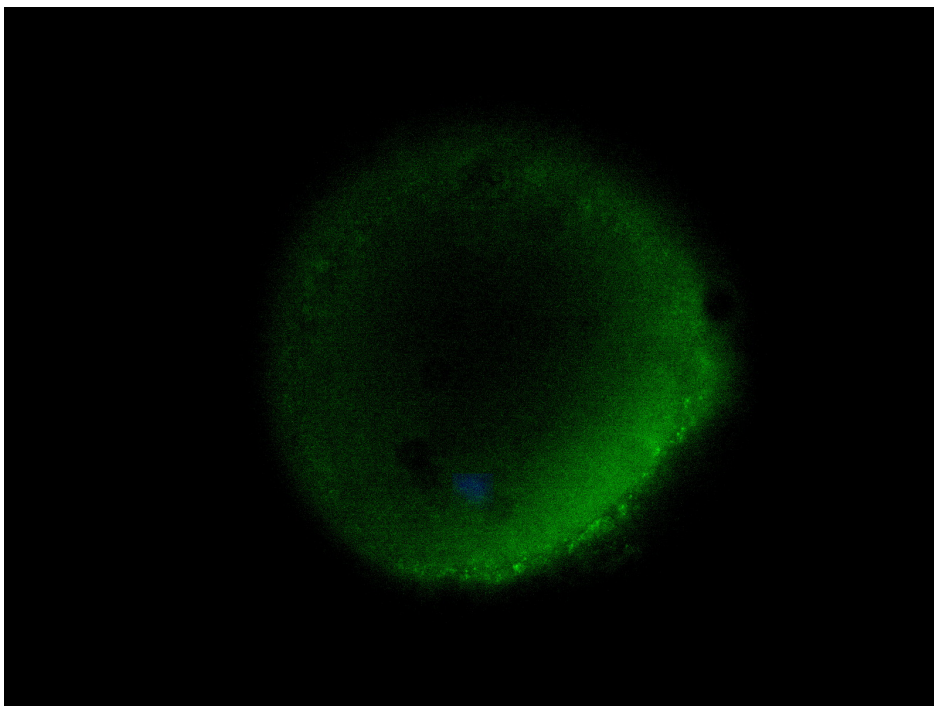
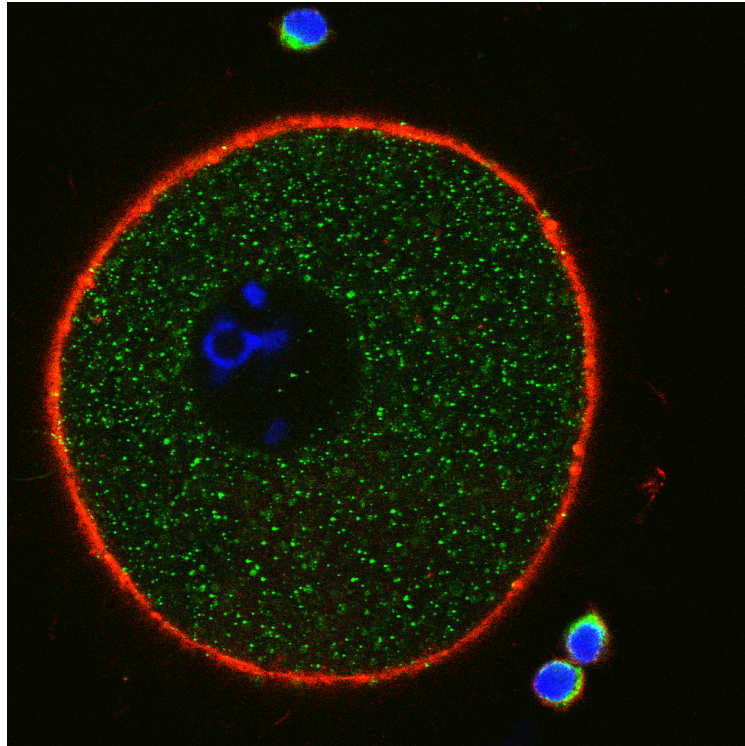


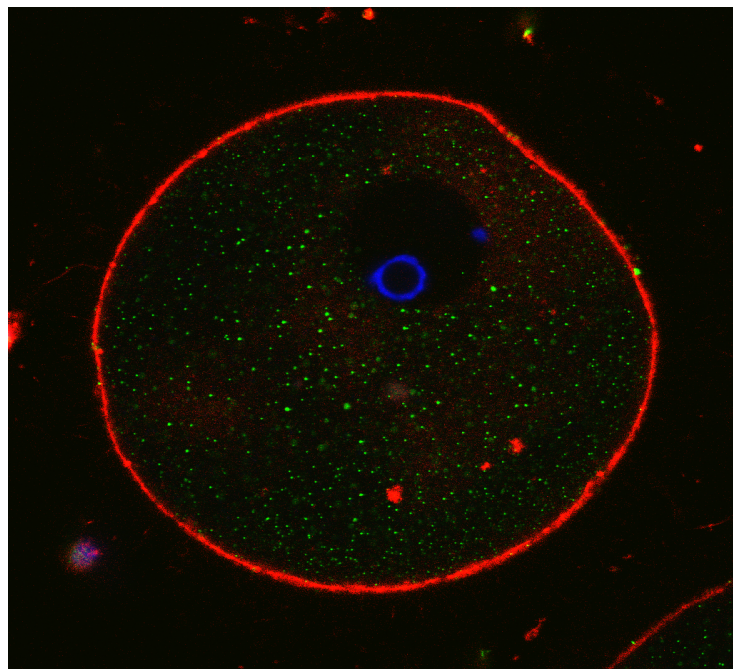
Figure 14: *Calreticulin* localization in a GV oocyte

Figures 15 A and 15 B represent the results obtained in immature GV oocytes respectively. Both images are representative of individual optical sections performed on the equatorial plane of each sample. The cellular margins are well defined from sub-olemmal actin (coloured in red) that has the role to provide strength to the cortical part of the oocyte, anchor the organelles such as cortical granules and ultimately assist the oocyte in the processes of polar bodies emission during meiotic maturation. In an intermediate position between the centre and the cortex it is well visible the nucleus (the GV) inside of which exclusively blue staining (specific for the chromatin) is detectable. The material does not show a diffuse and random chromatin distribution, but appears predominantly and specifically condensed around the nucleolus, according to a configuration defined as "surrounded nucleolus" (Figure 15 C). The organization of chromatin in GV is not the subject of this discussion, but it is known that it plays a specific functional role with respect to the ability of oocyte development. Overall, the cytoplasm is marked by a green staining characterized by a punctiform or finely granular distribution corresponding to the localization of CALR. From this staining it is possible to infer that the CALR is evenly distributed throughout the cytoplasm and closely surrounds the nucleus although being excluded (Figure 15 B), confirming its connection with the RE.

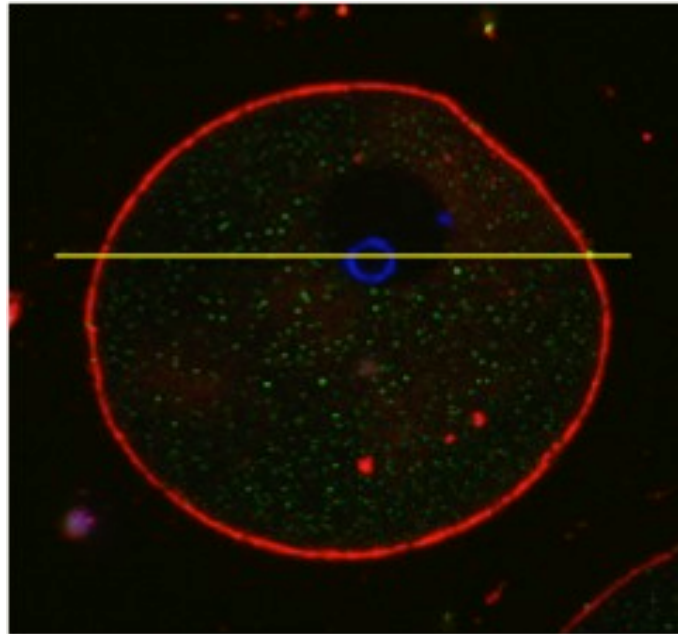
A



B



C



D

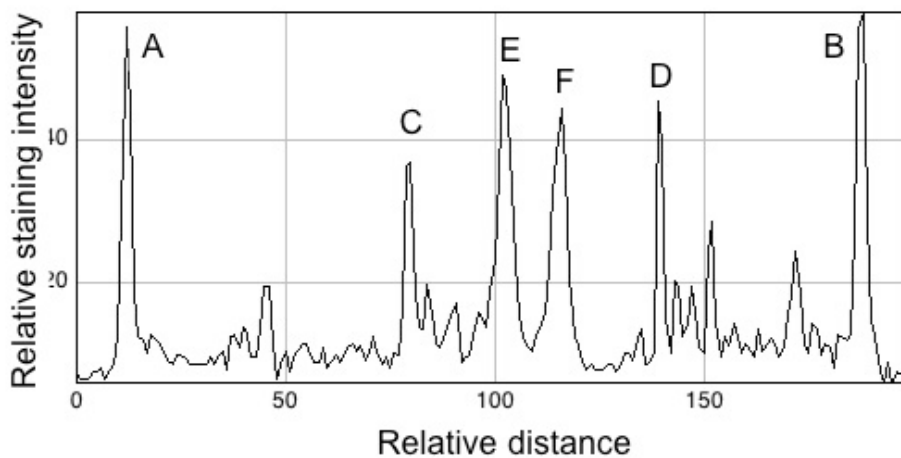
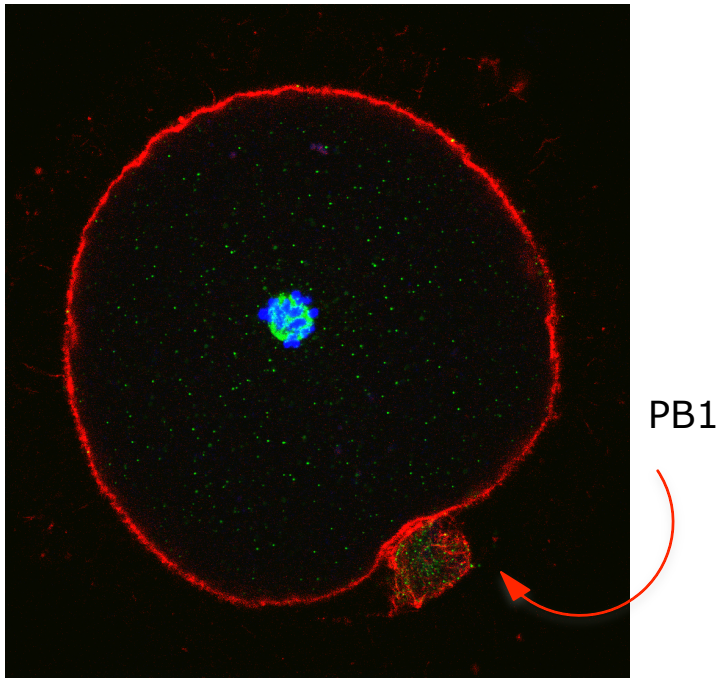


Figure 15: A and B: human oocytes at the GV stage stained by anti-*Calreticulin* antibody.¹⁵ C: Linear scanning of the confocal image of an oocyte at GV stage (top) stained for actin (red), DNA (blue) and CALR (green). In the graph D, the letter A and B represent the oocyte borders, C and D the limits of the nucleus (GV) and E and F denote the ring of chromatin surrounding the nucleolus. CALR signal is excluded from the nucleus and relatively weak.

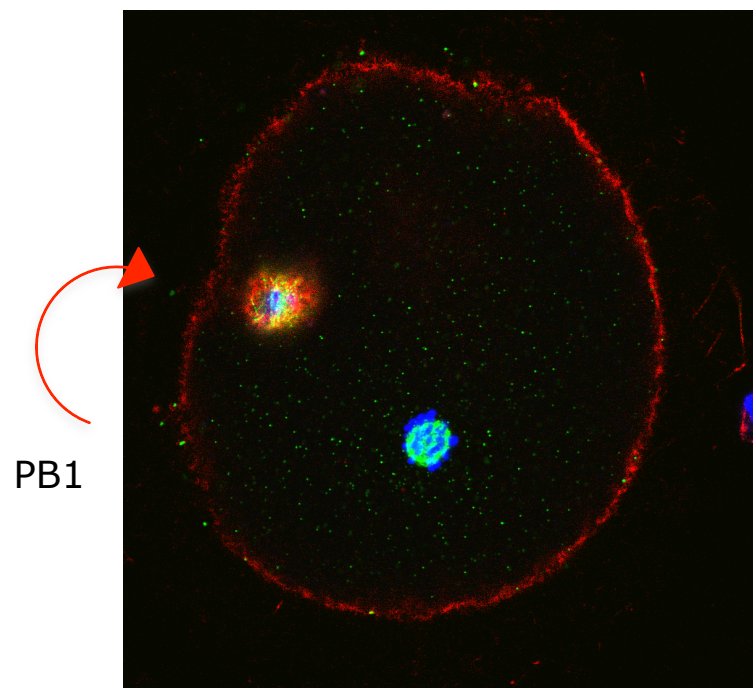
Figure 16 A and B are indicative of the patterns obtained with oocytes at the MII stage. In the left Figure, the staining for actin (red), in addition to outline the edge of the egg shows the first polar body in the "5 o'clock" position. The chromosomes are in a condensed status (in blue) are located centrally, probably held together by a microtubular structure organized in a meiotic spindle (not shown by the staining to which the oocytes were subjected). The chromosomes are abnormally far from the cortex to which they are usually anchored. The green staining, corresponding to the CALR, is detectable in a weakly diffused form and characterized by small dots throughout the cytoplasm. At the same time CALR is concentrated in the immediate vicinity of the chromosomes where it assumes a morphology that is highly suggestive of the meiotic spindle. The Figure on the right shows a very similar image in an MII stage oocyte where the first polar body is not perfectly distinguishable because occupying a position in a different geometric plane. However, and once again, the chromosomes (in a condensed state) are confined in a small area away from the cortex. The staining for CALR appears homogenous and weak throughout the cytoplasm, while it is intensely and specifically localized around the chromosomes with a morphology reminiscent of the meiotic spindle. The details shown

in the lower right image shows even more clearly the chromosomes and the distribution of the CALR (Figure 16 C).

A



B



C

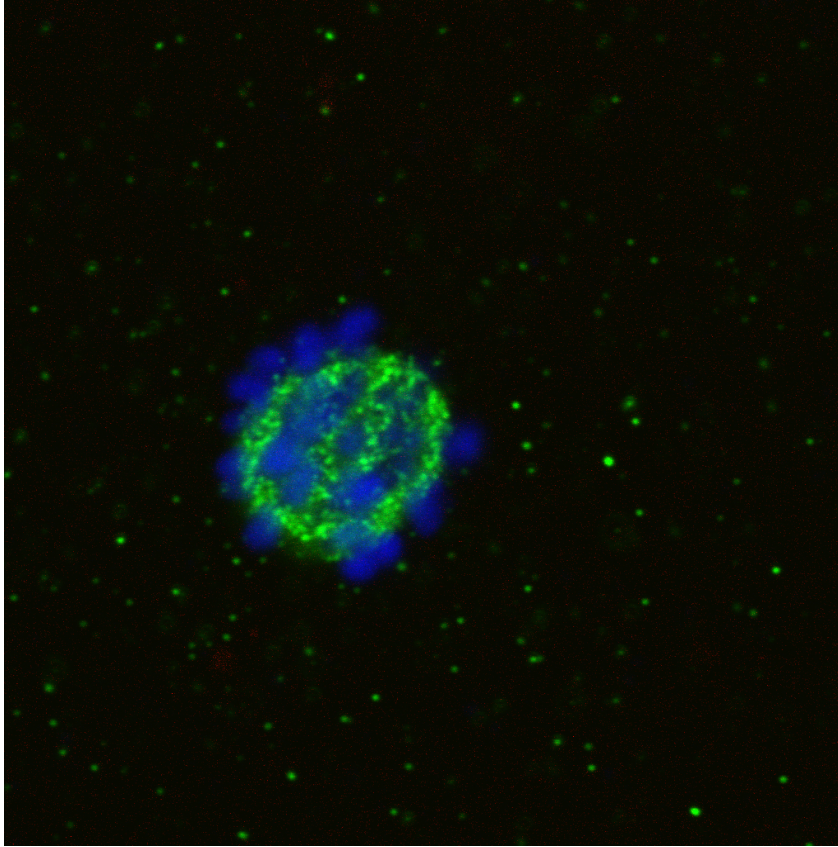
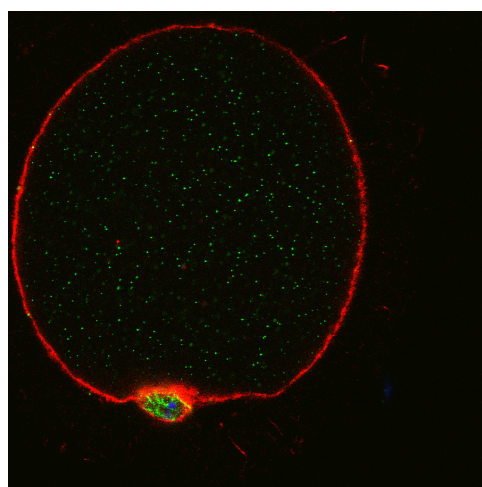


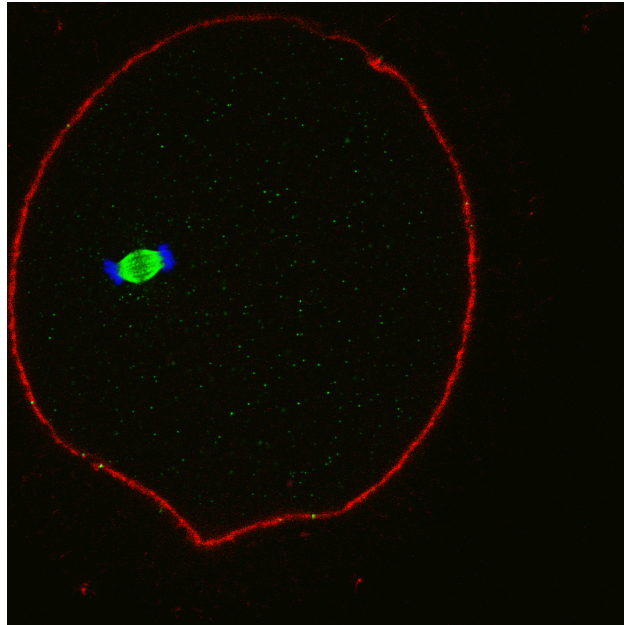
Figure 16 A and B: Human oocytes at the MII stage. In A the first polar body (PB1)(see red arrows) is evident at the 5 o'clock position and in B the PB1 remains on another observational plane. 16 C represents a particular of the CALR staining with the detail of chromosomes with respect to CALR localization

Figure 17 A and B describe the state of an oocyte observed on two distinct focal planes. In the left image it may be observed the first polar body in the typical 6 o'clock position and a weak and diffuse green staining specific for CALR. The right image shows the same oocyte observed on a different focal plane. Chromosomes are detectable and arranged in separate groups according to a pattern that resembles the stage of anaphase. This suggests that, prior to fixation, the oocyte has undergone a partial event of spontaneous activation in the absence of extrusion of the second polar body. The green staining specific for CALR, in addition to the usual diffuse distribution, assumes a distribution extraordinarily similar to the morphology of the meiotic spindle in correspondence of the area that separates the two sets of chromosomes. The detail picture shows even more clearly the analogy between the distribution of the CALR and the assumed morphology of the spindle (Figure 17 C1 and C2)

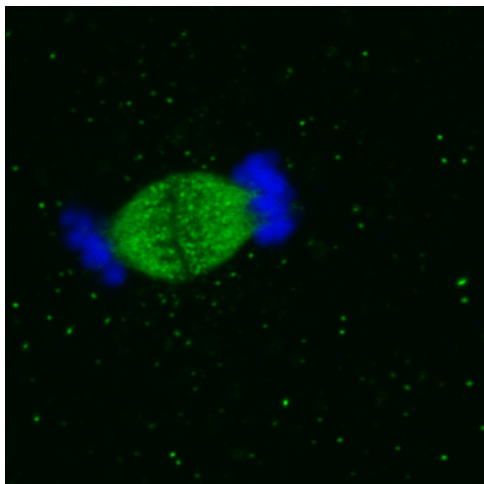
A



B



C1



C2

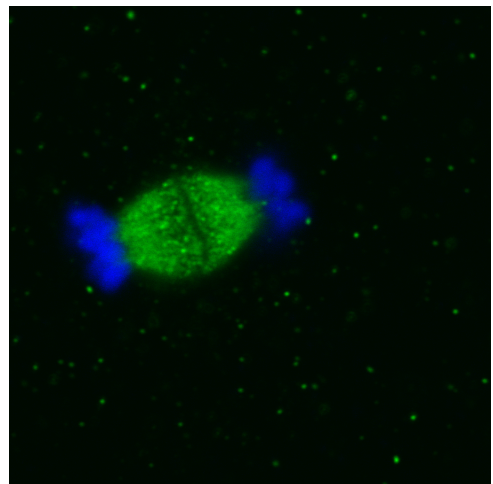
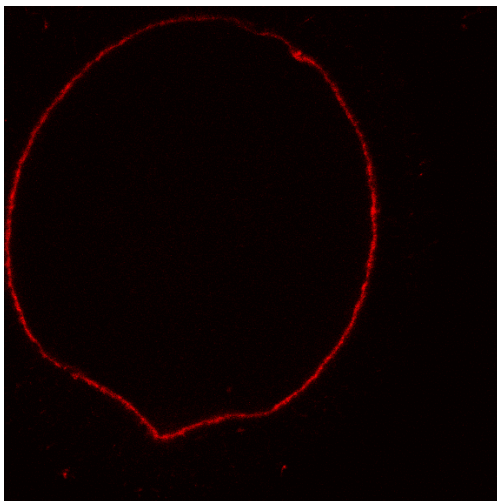


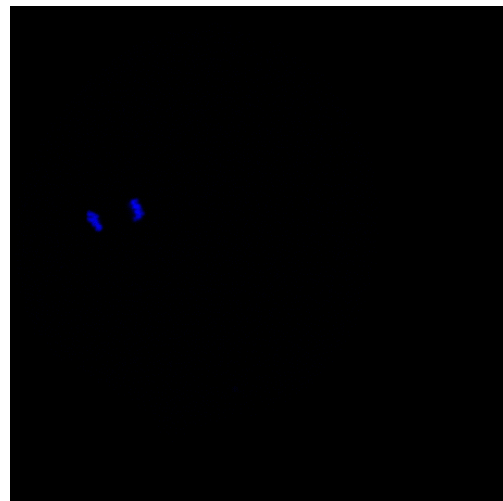
Figure 17 A and B: Human oocyte at the MII stage observed on two different focal planes. A with PB1 at "6" h position and B with PB barely distinguishable on a different focal plane but with chromosomes neatly arranged at the periphery of a structure evocative of the meiotic spindle. C1 and C2 represent details showing similarity between the pattern distribution of CALR and the assumed morphology of the meiotic spindle.

A really intriguing perspective comes from the observation of the images 17 B and C acquired for a single channel (corresponding to a single colour and in consequence a single target structure). In Figure 18 A only actin (red), in B only chromatin (blue) and in C only CALR (green) staining is acquired. Focusing on what presumably represents the meiotic spindle, although tubulin is not stained, it is evident that the chromosomes have moved to the poles of the spindle structure and CALR co-localise with the position of microtubules (probably around them) but is absent in the area that constitutes what was the metaphase plate of the first meiotic division. This is in agreement with the hypothesis that the oocyte analysed, although presenting an extruded first polar body, was still in telophase I (18 E).

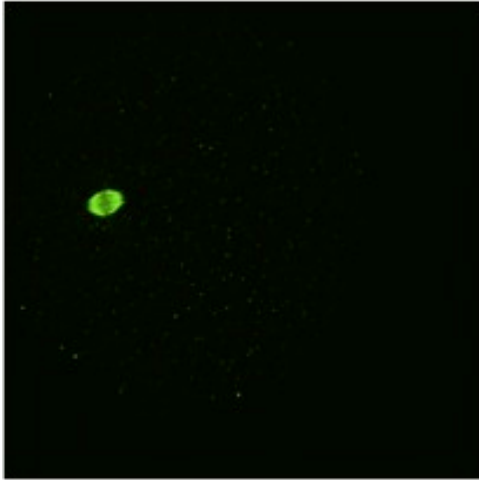
A



B



C



D

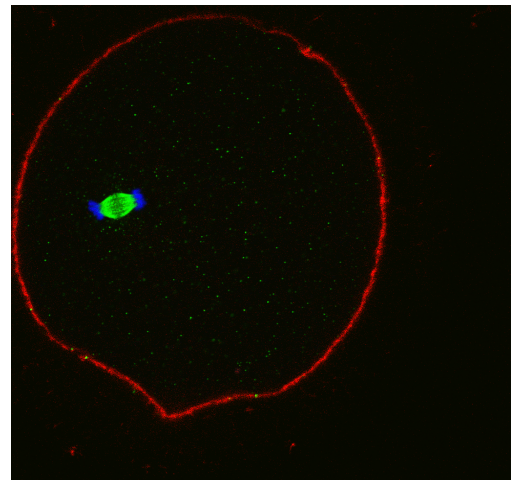


Figure.18 A, B and C represent the same Anaphase/Telophase I oocyte stained for actin, DNA and CALR respectively, merging picture Figure 17 B or 18 D

E

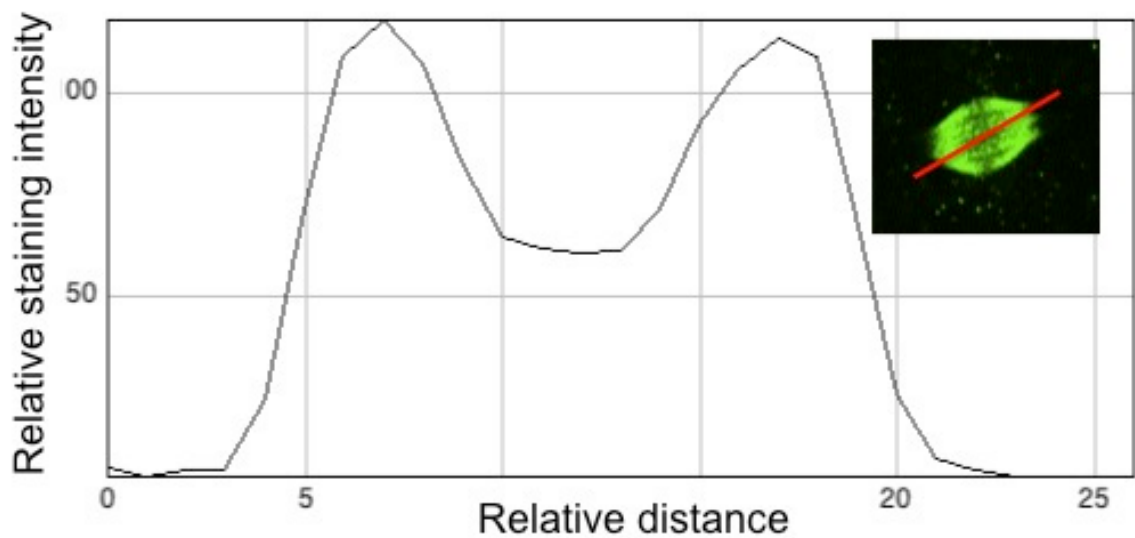


Figure 18 E: Graphic representation of CALR staining intensity co-localized with the meiotic spindle. Inset shows the line along which the staining intensity was measured.

5. DISCUSSION

The oocyte undergoes a remarkably long and complex excursion prior to reaching the MII in a condition that renders it fully competent for fertilization. A multitude of processes prepares the oocyte for fertilization; thus a deficiency in any one or more event(s) will render the oocyte unsuitable for fertilization. All of the preparatory events occur during the protracted timeline of oogenesis within the unique and highly differentiated background created by the follicle. A large body of evidence has demonstrated the determining influences of the differentiation state of the follicle on the quality of its enclosed oocyte. Follicle development is a characteristically dynamic process; in a cyclic fashion, follicles grow and differentiate as a group or cohort while a single follicle will be selected and continue development as the dominant or pre-ovulatory one (Combelles and Rawe, 2013). Consequently, all follicles within a recruited cohort (other than the dominant one) are not naturally selected for ovulation; it is thus not surprising that during routine IVF, oocytes are retrieved from some follicles that have not completed the developmental program necessary to support an oocyte to full competence, however the possibility to obtain spare oocytes from patients undergoing IVF treatments for

infertility allows scientists to obtain information of paramount importance in the field of reproductive biology.

The growing oocyte also experiences a remarkable accumulation of ribosomes, RNA transcripts, and proteins, some of which are required for the continued differentiation of the oocyte itself, and others for fertilization. Through the growth phase, the oocyte becomes meiotically competent or able to resume meiosis in a stepwise manner. Depending on the species, meiotic competence may not be attained until antral folliculogenesis, a period during which the oocyte will continue acquiring developmental competence. Many of the modifications taking place during oocyte growth are obligatory for later differentiation events when the oocyte becomes endowed with further specializations for fertilization. The developmental phase prior to meiotic resumption is referred to as oocyte capacitation or pre-maturation. Oocyte maturation entails molecular events during which transcripts and proteins are stockpiled in the ooplasm, in anticipation for their use during fertilization and later development. Indeed, a recurring theme is the accumulation of cytoplasmic components while the oocyte is progressing through maturation, such that by the time MII is reached, the majority of cytoplasmic events are completed. This has been shown for the ability to sustain calcium oscillatory patterns (Jones *et al.*, 1995) and the reorganization of ER clusters in the oocyte cortex (FitzHarris *et al.*, 2007).

Finally, asymmetry of oocyte meiotic divisions allows preservation of maternal mRNAs, proteins and nutrients in the cytoplasm, accumulated during the growth phase of oogenesis (Verlhac and Breuer, 2013).

In this perspective, cytoplasmic RNA transport is a powerful mechanism for spatially restricting protein expression (Palacios and St Johnston, 2001). Within germ cells, mRNAs are actively transported to specific subcellular destinations, which is an important mechanism to establish regulated cellular asymmetry of gene expression during differentiation and development. Developmental polarity can be achieved by localization of maternal determinants in the form of mRNA. In recent years, much progress has been made toward understanding the molecular mechanisms underlying the process of RNA localization through studies in a variety of systems. It has become clear that localized RNAs carry localization signals that interact with trans-acting factors to direct localization. Many of these factors have been identified (Jansen, 2001), but few are conserved among the many organisms studied. A lack of conservation of RNA transport machineries could suggest that mechanistic strategies for RNA localization vary among different organisms. However, one localization factor does appear to be conserved among different organisms; *Staufen* may be the first RNA-binding protein to provide a mechanistic link between invertebrates and vertebrates in RNA localization, but its involvement in mammalian species, included the human, remains to be established. mRNAs can be

delivered to the correct destination in a variety of ways, such as local protection from degradation, diffusion and anchoring, or active transport along either the actin or microtubule cytoskeletons. One of the best-characterized systems for studying the latter mechanism is the formation of the anterior–posterior axis in *Drosophila*, which is specified by the microtubule-dependent localization of bicoid mRNA to the anterior of the oocyte and of oskar mRNA to the posterior (Bastock and St Johnston, 2008). bicoid mRNA is translated at the anterior after egg activation to produce a protein gradient that acts as a morphogen to pattern the head and thorax of the embryo (Ephrussi and St Johnston, 2004). oskar mRNA, on the other hand, is translated once it is localized to the posterior pole of the oocyte, where Oskar protein defines the site of pole plasm assembly, leading to the posterior recruitment of the abdominal determinant, nanos RNA (Ephrussi *et al.*, 1991) (Kim-Ha *et al.*, 1991). Mutants that disrupt the localization of bicoid mRNA produce embryos with defective heads, whereas oskar mRNA localization mutants result in embryos without pole cells or an abdomen. The localization of bicoid mRNA also occurs in multiple steps during oogenesis, although genetic screens have been less successful at identifying the necessary factors. Like oskar, bicoid mRNA is transcribed in the nurse cells and transported by dynein into the oocyte (Clark *et al.*, 2007). It is then localized to the anterior cortex of the oocyte. Several additional proteins are required to keep bicoid mRNA at the

anterior of the oocyte after stage 10a of oogenesis. Although the RNA-binding proteins that recognize bicoid mRNA to mediate early localization have not been identified, these latter stages require *Staufen* which binds specifically to a region of the bicoid 3' UTR (Ferrandon *et al.*, 1994). The localization of bicoid mRNA changes again in mature oocytes, and the RNA becomes stably anchored to the actin cortex until the egg is activated, which releases bicoid mRNA/*Staufen* complexes into the egg cytoplasm and activates Bicoid translation (Weil *et al.*, 2008). Miranda binds directly to the C-terminal domain of *Staufen* to localize *Staufen/prospero* mRNA complexes to the basal cortex of the neuroblast (Broadus *et al.*, 1998) (Matsuzaki *et al.*, 1998) (Shen *et al.*, 1998) and generally is not expressed in the female germline, but binds to *Staufen/oskar* mRNA complexes when expressed ectopically and localizes with them to both the anterior and posterior of the oocyte (Chang *et al.*, 2011). In *Xenopus laevis*, Yoon and colleagues (Yoon and Mowry, 2004) identified a homolog of *Staufen* (XStau) and provided evidence of a role in RNA localization during frog oogenesis. XStau interacts with specific localized RNAs during oogenesis and is expressed in a spatial and temporal pattern consistent with a role in RNA localization. Moreover the Authors showed that a mutant version of XStau blocks RNA localization in oocytes suggesting that XStau is an integral component of the machinery necessary to localize RNA to the vegetal cortex and that may mediate interactions between localized

RNAs and motor proteins that are crucial for transport. In *Xenopus laevis* during oogenesis, maternal RNAs localize to the vegetal cortex of the oocyte and confer germ layer determination and germline specification during later embryonic development. Vegetally localized RNAs can be placed into two classes depending on their timing and mechanism of transport. Maternal mRNAs that are localized by the early localization pathway, accumulate in the mitochondrial cloud at the beginning of oogenesis (stage I) and arrive at the vegetal cortex by stage II. The second pathway that functions to localize mRNAs to the vegetal pole was largely defined through studies on Vg1 RNA (Forristall *et al.*, 1995). RNAs localized by the late pathway are uniformly dispersed in the cytoplasm of stage I oocytes. During mid-oogenesis, the RNAs are transported to the vegetal cortical cytoplasm, where they remain tightly anchored to the vegetal cortex until the end of oogenesis (stage VI). RNA localization relies on cis-acting sequence elements within the RNA that are recognized by trans-acting components of the localization machinery (Jansen, 2001). These cis-elements generally reside within the 3' untranslated region (UTR) of the localized message and are essential for proper transport. However, the many trans-factors identified are generally non-homologous between vertebrates and invertebrates. This raises the issue of whether the mechanistic strategies for RNA localization are shared among organisms. One factor that could mechanistically link RNA localization in vertebrates and

invertebrates is the double-stranded RNA (dsRNA)-binding protein *Staufen*. Since the identification of *Drosophila Staufen*, several vertebrate homologs have been found (Kiebler *et al.*, 1999; Marion *et al.*, 1999; Wickham *et al.*, 1999). Nonetheless, the functions of known vertebrate *Staufen* homologs are not yet clear. Mammalian *Staufen* has been shown to colocalize with polyribosomes and rough endoplasmic reticulum in cultured cells and hippocampal neurons suggesting a role in RNA localization. However, interactions with specific localized RNAs have not been uncovered, leaving open the question of whether this RNA-binding protein may assist a conserved role in RNA localization. With respect to *Staufen* localization, although in *Drosophila* and *Xenopus*, it is characterized by a specific polarized pattern, in large animals, like the pig, the localization of *Staufen* results rather specific but not polarized changing distribution during the progression from GV to MII. The pig orthologue for *Staufen* has been identified in 2005 (*Staufen*; EMBL accession number: AJ969068) by Brevini (Brevini *et al.*, 2005). Pig *Staufen* shows a 90% homology with the human sequence but only 32% with the *Drosophila* orthologue. The study conducted in pig oocytes found that *Staufen* is a maternally expressed gene in the pig since it was possible to consistently detect its transcript during *in vitro* maturation. Immunolocalization studies, performed using an antibody raised against a synthetic peptide corresponding to amino acids 503–617 of the human *Staufen*, demonstrated a specific

cytoplasmic localization of the protein during *in vitro* maturation. A fine, diffuse distribution of *Staufen* was visible in GV oocytes. As maturation progressed, the signal for *Staufen* became more granulated and seemed to rearrange in a more restricted distribution at the inner and cortical regions of the oocyte. MII pig oocytes displayed large, discrete *Staufen-positive* granules distributed throughout the cytoplasm with an empty cone corresponding to the meiotic spindle. This pattern of distribution described for *Staufen* closely resembles the reorganization of endoplasmic reticulum that takes place during pig, mouse and hamster oocyte maturation (Cran, 1985; Shiraishi *et al.*, 1995) (Mehlmann *et al.*, 1996). From the above description, it appears that *Staufen* is present in a variety of organisms. However, its role and involvement in mRNA regulation has probably changed during evolution. In invertebrates (*Drosophila*), *Staufen* is implicated in mRNA transport and localization and, importantly, carries out this role in a sequence-specific fashion, because only a few selected mRNAs are recognized and regulated. Such a mode of action results particularly important for species such as the *Drosophila* in which the general body plan of the developing organism is dictated by cytoplasmic determinants laid during oogenesis. In general, this role of *Staufen* results to be conserved in lower vertebrates where, although to a lesser extent, the specific localization of cytoplasmic determinants produced during oogenesis contribute to design the embryo developmental strategy. In mammalian

oocytes, regional confinement of cytoplasmic determinants does not appear to be a dominant developmental strategy, with the implication that the regulative significance of *Staufen* may be different. The evidence generated in the pig does not support the hypothesis that *Staufen* regulates mRNA function recognizing specific cis-acting sequences. In fact, as described above, *Staufen* distribution changes during maturation, but widespread localization in the cytoplasm suggests against an action directed towards specific mRNAs. It is possible that during evolution *Staufen* may have lost the ability to regulate individual types of mRNA by recognizing specific cis-acting sequences, but may have retained the capacity to bind and transport mRNA relatively indiscriminately, from the site of production (the nucleus) to the sites of translation. The fact that the transition of *Staufen* distribution during maturation mirrors that of the ER may be interpreted as a clue in agreement with this hypothesis. In human oocytes, we have observed a changing pattern of *Staufen* distribution that is reminiscent of the one occurring in the pig, confirming that in mammals this protein is involved in regulative mechanisms that are different from those characterizing lower species, such as the fly and the frog. Following up the lead of the similarity between *Staufen* and ER changes in distribution during maturation, the present work focussed also at monitoring the pattern of localization of CALR, as marker for the localization of the ER. The results collected shows that at the GV stage,

CALR distribution appears finely granular and ubiquitous throughout the cytoplasm, with a moderate accumulation around the GV. This pattern is similar to the one of *Staufen* observed at the same stage of maturation. However, contrary to the case of *Staufen*, in MII stage oocytes CALR localization does acquire the pattern of larger granules with a non-regionalized distribution. Rather CALR staining appears concentrated and coincident with the presumptive position and morphology of the meiotic spindle, as confirmed also by the relative position of the chromosomes. Although this finding was not expected, it does not exclude the possibility that *Staufen* dynamics reflects the one of ER in order to assist the process of translation. More simply, it is possible that CALR, while being a very important component of the ER, is segregated in a specific ER subdomain that interacts with the meiotic spindle. In fact, at least in one case (the mouse) it has been shown that at the MII stage the ER acquires a highly specific conformation enveloping the meiotic spindle (John Carroll, personal communication see Figure 19). The significance of such an association is not fully understood, but it is plausible that the dense microtubular array forming the meiotic spindle is locally and specifically regulated by Ca^{2+} ions released from a subdomain of the ER selectively enriched in CALR.

In conclusion, the present data support the notion that *Staufen* distribution undergoes a specific rearrangement during meiotic maturation in the human oocyte. The specific patterns observed at the

different maturation stage are compatible with an involvement in the general process of mRNA translation. Less evident, according to the data collected in the present work, is a possible implication of *Staufen* in the localization of specific mRNAs. This would differentiate the role of *Staufen* in mammals, in comparison to invertebrates and amphibians. Finally, CALR specific localization in association with the meiotic spindle suggests the hypothesis of different ER subdomains and an important involvement of Ca^{2+} in the regulation of the meiotic spindle. This opens entirely new scenarios for future studies on the ER-cytoskeleton physical and regulative interaction in the human oocyte.

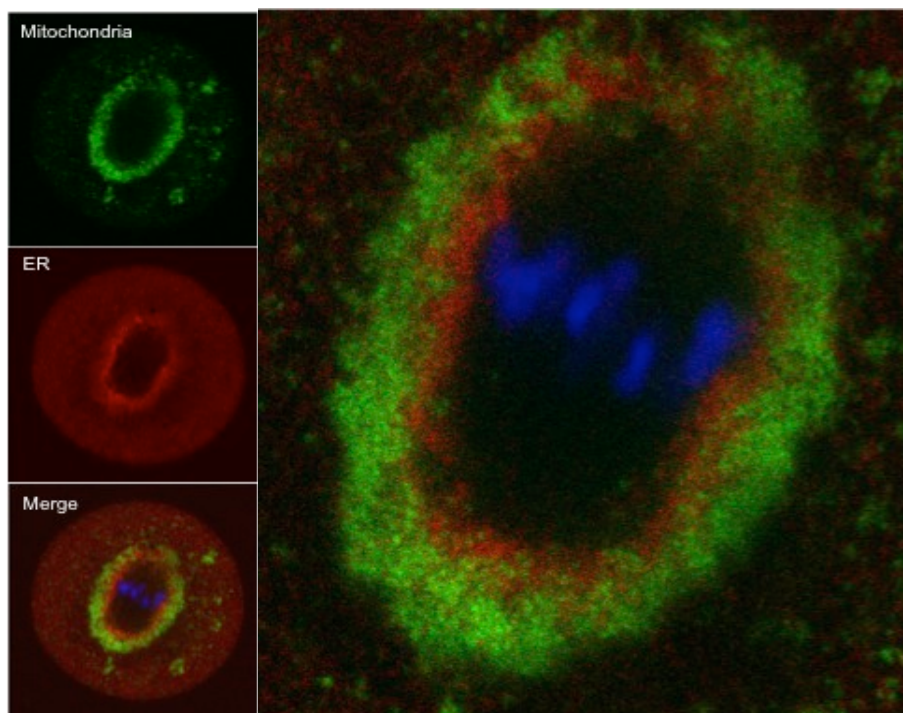


Figure 19: A mouse oocyte showing a meiotic spindle stained for Mitochondria (GREEN), ER (RED) and Chromosomes (BLUE). Courtesy of John Carrol

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