An increasing number of research highlights the crucial role of vitamin D_3 in the regulation of reproductive processes in females. It has been shown so far that the mammalian ovary express vitamin D_3 receptors and it is also an important extrarenal site of vitamin D_3 metabolism. In recent years, a global problem of vitamin D_3 deficiency has been observed in the human population, which is associated with the pathogenesis of many diseases, including ovarian dysfunctions, *i.e.* polycystic ovary syndrome (PCOS). One of the PCOS symptoms are metabolic disorders such as insulin resistance and hyperinsulinemia. It has been shown that vitamin D_3 supplementation can increase insulin sensitivity of peripheral tissues, which is promising in the treatment of PCOS. Considering the direct effect of vitamin D_3 on insulin secretion and the fact that vitamin D_3 and insulin are important regulators of physiological and pathological processes in the ovary, the question arises regarding their mutual interactions at the ovary level. This problem has not been studied so far, therefore the goal of this study was to demonstrate the molecular mechanism of vitamin D_3 and insulin action in the ovarian follicle by global analysis of its proteome, including granulosa cells, theca interna cells and extracellular vesicles (EVs) isolated from follicular fluid. This study was conducted using the pig as an experimental model. Medium antral follicles were isolated from the ovaries of sexually mature pigs. Ovarian follicles were subjected to 12-hour incubation in control conditions (group C) and with the tested compounds: vitamin D_3 (group VD; 100 ng/ml), insulin (group I; 10 ng/ml), and vitamin D_3 and insulin together (group VD+I). After incubation, the granulosa cell layer and theca interna layer of the ovarian follicle were mechanically separated. The same experiment was conducted to collect follicular fluid for EVs isolation with size exclusion chromatography. The concentration and size distribution of EVs were determined using nanoparticle tracking analysis and the zeta potential analysis using electrophoretic light scattering, the morphology of EVs was assessed using transmission electron microscopy, and their phenotypic analysis was performed using flow cytometry. The samples of granulosa cells, theca interna cells, and EVs were subjected for protein isolation and quantitative proteomic analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) coupled with tandem mass tagging (TMT). To identify differentially abundant proteins (DAPs), the comparative bioinformatic analysis was performed in R Studio (t-test with B-H FDR; fold change = ±1.5; q-value < 0.05) followed by functional analyses using g:Profiler, KOBAS and STRING (B-H FDR; p-value < 0.05). Additionally, validation of results obtained on granulosa and theca interna cells was conducted using Western blot, proliferation assay, flow cytometry, transmission electron microscopy and immunofluorescence. In the granulosa layer, the comparative bioinformatic analysis identified 97 DAPs between groups: 14 DAPs in the

VD *vs* C comparison, 5 DAPs in the I *vs* C comparison, 44 DAPs in the VD+I *vs* C comparison, 57 DAPs in the VD *vs* VD+I comparison, and 5 DAPs in the I *vs* VD+I comparison. Vitamin D_3 affected the proteome of granulosa cells, inducing changes in the expression of proteins promoting their proliferation. In the VD+I group, however, DAPs were mainly involved in the regulation of the cell cycle. These results were confirmed by the proliferation assay and flow cytometry, indicating that vitamin D_3 alone, as well as the in co-treatment with insulin, increased granulosa cell proliferation with a simultaneous increase in the number of cells in the S phase of the cell cycle, in which DNA synthesis occurs. In the theca interna layer, the comparative bioinformatic analysis identified 11 DAPs between groups: 4 DAPs in the VD *vs* C comparison, 8 DAPs in the VD+I *vs* C comparison, and 2 DAPs in the I *vs* VD+I comparison. Functional analysis of DAPs showed their annotation to cholesterol transport, and lipid and steroid metabolic processes. In the VD group, a reduced expression of the SCARB1 protein, a membrane receptor of high-density lipoproteins, was demonstrated that correlated with a reduced number of lipid droplets in theca interna cells. These results suggest the influence of vitamin D_3 on cholesterol uptake and thus the regulation of steroid hormone biosynthesis. The characterization of EVs derived from follicular fluid showed that insulin reduced the concentration and mean average size of EVs, while vitamin D_3 reversed only its effect on the EV concentration. This may indicate the role of vitamin D_3 and insulin on EV biosynthesis and release into the follicular fluid. Proteomic analysis of EV proteome revealed 48 DAPs between groups: 19 DAPs in the VD *vs* C comparison, 16 DAPs in the I *vs* C comparison, 8 DAPs in the VD+I *vs* C comparison, 8 DAPs in the VD *vs* VD+I comparison and 10 DAPs in the I *vs* VD+I comparison. Based on the functional analysis of DAPs, vitamin D_3 alone or in co-treatment with insulin has been shown to change the expression of ribosomal proteins, which may constitute a new element of communication within the ovarian follicle. Insulin, on the other hand, affected the expression of proteins involved in oxidative stress. Analysis of DAPs in the VD+I *vs* C comparison revealed increased expression of adiponectin, which may be a new marker of vitamin D_3 and insulin interaction at the ovarian level, suggesting an increased sensitivity of this tissue to insulin in the presence of vitamin D_3. Therefore, vitamin D_3 and insulin may regulate ovarian functions *via* an EVs-dependent pathway. Concluding, the analysis of the global proteome of the ovarian follicle in a pig model has shown that vitamin D_3 and insulin co-treatment induce the most prominent changes in the proteomic profile of granulosa cells and theca interna cells. They indicate the regulation of processes important for ovarian function, including granulosa cell proliferation and cholesterol transport into theca interna cells. This thesis presents for the first time the role of vitamin D_3

and insulin interactions in the EV biogenesis and release into follicular fluid in the porcine ovary. The influence of the studied compounds on the expression of proteins carried by EVs, which participate in biological processes important for ovarian functions, has also been demonstrated, including these changes that may have broader implications in ovarian pathologies. A comprehensive presentation of the molecular mechanisms of vitamin D_3 and insulin action within the porcine ovarian follicle may contribute to a better understanding of biological processes occurring in the ovary under physiological and pathological conditions.