Impaired testicular signaling of vitamin A and vitamin K contributes to the aberrant composition of the extracellular matrix in idiopathic germ cell aplasia

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Objective: To study pathogenic features of the somatic testicular microenvironment associated with idiopathic germ cell aplasia.
Design: Cross-sectional study.
Setting: Tertiary referral center for reproductive medicine.
Patient(s): Testicular specimens from men with idiopathic nonobstructive azoospermia (iNOA) prospectively submitted to microdissection testicular sperm extraction. Of 20 specimens used for histology, 10 were also available for proteomic analysis. Primary Sertoli cells with normal karyotype and phenotype were also used.
Intervention(s): Patients with iNOA were dichotomized according to a positive versus negative sperm retrieval at microdissection testicular sperm extraction, and on the isolated extracellular matrix (ECM) the proteomic analysis was performed.
Main Outcome Measure(s): Proteomic analysis of the ECM from testicular specimens with positive versus negative sperm retrieval. Gene ontology enrichment was used to identify upstream regulators based on the 11 deregulated ECM proteins, which were validated by immunohistochemistry and quantitative polymerase chain reaction. Continuous variables were expressed as medians and interquartile range.
Result(s): Germ cell aplasia was characterized by an increased signaling of the retinoic acid in Sertoli cells and associated with decreased expression of the basal membrane markers nidogen-2 and heparan sulfate proteoglycan-2. Decreased levels of the interstitial matrisome-associated factor IX and its regulator VKORC1 were, instead, coupled with decreased signaling of vitamin K in Leydig cells. An altered expression of a further eight ECM proteins was also found, including laminin-4 and laminin-5. Peripheral levels of the two vitamins were within the reference range in the two cohorts of iNOA men.
Conclusion(s): We identified the pathogenetic signature of the somatic human testicular microenvironment, providing two vitamin-related mechanistic insights related to the molecular determinants of the idiopathic germ cell aplasia. (Fertil Steril® 2019;111:687–98.
El resumen está disponible en Español al final del artículo.

Key Words: Extracellular matrix, vitamins, male infertility, azoospermia, testis

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Several studies have established the relevance of the somatic microenvironment in supporting spermatogonial stem cell differentiation. Indeed, germ cells of an infertile man (1) or an aged mouse (2) retain their capability of generating functional spermatozoa for a long time when, upon transplantation into a healthy or younger recipient's testis, they mature into functional spermatogonia. Conversely, spermatogonial stem cells from young mice transplanted into atrophic testicles are unable to promote and support spermatogenesis (3). Similarly, preventing the aging of the somatic environment counteracts the loss of spermatogonial stem cells in aged *Drosophila melanogaster* (4).

The extracellular matrix (ECM) represents the most important component of the somatic microenvironment (5). Analysis of autoptic human tissues revealed that the testes contain the largest number of tissue-enriched genes, many of them involved in spermatogenesis and reproduction (6, 7); likewise, the testis contains 102 proteins ascribed to the ECM (8), whose role in supporting human spermatogonial stem cell survival was recently reported (9).

In this study, we uncovered several pathophysiological features of the somatic testicular microenvironment that are associated with human germ cell aplasia and that eventually lead to idiopathic nonobstructive azoospermia (iNOA), the most severe form of male factor infertility (10). With this in mind, testicular specimens collected by microdissection testicular sperm extraction (microTESE) from iNOA men where dichotomized into sperm retrieval-positive (SRpos) versus sperm retrieval-negative (SRneg, consistent with the histological diagnosis of Sertoli cell only syndrome (SCOS) and complete germ cell aplasia according to the surgical outcome. Therefore, testis ECM composition was assessed in both groups, and gene ontology enrichment was used to evaluate pathogenic pathways associated with germ cell aplasia.

MATERIALS AND METHODS

Ethics Approval

Data collection followed the principles outlined in the Declaration of Helsinki; all patients signed an informed consent agreeing to supply their own anonymous information and tissue specimens. The study was approved by the Institutional Review Board (authorization protocol URI001-2010, Ethic Committee of IRCCS Ospedale San Raffaele, Milan, Italy).

Study Population

Testicular tissues and clinical data from 20 white-Caucasian men with iNOA submitted to microTESE between 2014 and 2016 at one tertiary referral center for reproductive medicine were analyzed. Infertility was defined as not conceiving a pregnancy after at least 12 months of unprotected intercourse (11), and men were classified as NOA when no spermatozoa were present in at least two consecutive semen analyses because of nonobstructive causes (12). Idiopathic NOA was determined following comprehensive diagnostic evaluations of all known causes of NOA, as we recently reported (13).

A venous blood sample was drawn between 7 a.m. and 11 a.m. after an overnight fast at the time of surgery, and hormones were measured as detailed (13). Serum level of retinol was assessed by HPLC (Chromsystems Instruments and Chemicals). The prothrombin ratio was measured by treating blood plasma with STA Neoplastin plus on the coagulometer STA-R (Roche) and was expressed as international normalized ratio.

MicroTESE, Sperm Retrieval, and Tissue Analysis

All 20 iNOA men underwent microTESE according to the original surgical technique (14). Sperm retrieval was immediately attempted on testis specimen (13), and patients were then dichotomized according to the surgical outcome (SRpos vs. SRneg, n = 10 in both groups).

Testicular specimens were also obtained from the non-neoplastic tissue of five men submitted to unilateral orchiectomy for nonmetastatic seminoma. Control tissue was obtained from the most distant area from the tumor and identified as having normal germ line cell maturation based on a thorough histological analysis. For each seminoma patient, preoperative semen cryopreservation excluded NOA in all cases.

All testicular specimens (20 iNOA and five controls) were formalin fixed and paraffin embedded (FFPE) for histological and immunohistochemical analyses, performed by experienced uropathologists. Morphological evaluations of Leydig cell compartments were also performed and are reported as [1] normal Leydig cells; [2] Leydig cell hyperplasia (multiple nodules, <0.5 cm in diameter, with proliferative Leydig cells); and [3] Leydig cell tumor (isolate, <3 cm in diameter, well delimitated solid nodule of proliferative atypical Leydig cells).

Immunohistochemistry staining was performed on FFPE slides of the testis according to a methodology described elsewhere (15). Primary antibodies against human antigens were anti-VKORC1 (clone HPA042720 validated in the Human Protein Atlas, Sigma-Aldrich), anti-calretinin (CALB2 clone 790-4467, Ventana Medical Systems), anti-factor IX (clone LS-B6248, LSBio), and anti-RAR-alpha (clone 2D2, MyBioSource). Negative control was obtained by omitting the primary Ab. Specificity of the immunohistochemistry signal was compared with that reported in the Human Protein Atlas (https://www.proteinatlas.org/) for human testis.

The number of Sertoli cells with RARα nuclear localization was counted in 10 tubule sections/each case: at least 150 Sertoli cells were analyzed for each case, and the results are presented as the number of RARα+ Sertoli cells/tubule section and the percentage of RARα+ cells versus the total number of Sertoli cells.

ECM Purification and Protein Identification

Testicular specimens were embedded in optimal cutting temperature (OCT) compound (Bio-Optica) and stored at −80°C. OCT-embedded tissue was available only for 10 iNOA men to be used for ECM purification and biochemical characterization, as recently described (16, 17) and detailed in the Supplemental Materials and Methods: ECM proteins were

compared to the Total Human Matrisome database (18). The mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium via the PRIDE (19) partner repository with the data set identifiers PXD011817 and 10.6019/PXD011817.

Phenotype and Karyotype Analysis of Primary Sertoli Cells

Human Sertoli cells (HSECs) from two Caucasian men (15 and 23 year old) were bought from Lonza, which certified cell purity by cytofluorimetric analysis (>97% SOX9+; >96% GATA4+). To verify that HSECs maintained their own functional status at passage 4, they were analyzed for the expression of SOX9, GATA4, and the Sertoli-specific claudin 11 and androgen receptor (AR). RNA from 50,000 HSECs at passage 4 was extracted using the RNeasy Plus Micro Kit (Qiagen) and retrotranscripted with a High Capacity RNA-to-cDNA Kit (Applied Biosystems Italia). Total DNA was amplified for the quantification of the copies of SOX9 (primer dHsaCPE5035865 HEX, Biorad), GATA4 (primer Hs00171403_m1 FAM, ThermoFisher), CLDN11 (primer Hs00194440_m1 FAM, ThermoFisher), and AR (Hs00171172_m1 FAM, ThermoFisher), using the digital droplet polymerase chain reaction (PCR) according to the manufacturer's instructions, as recently reported elsewhere (20). The number of copies was normalized to the total nanograms of loaded DNA. Furthermore, HSECs cultivated in Dulbecco's modified Eagle's medium/F12 added at 5% maintained proliferative activity up to eight passages, when they reached a plateau, and at passage 11 the proliferation activity decreased. Metaphase chromosome preparation was obtained from HSECs as reported (21).

Genetic Assessment of HSECs

HSECs were screened for genetic abnormalities that have been previously associated with azoospermia: homo- and heterozygosis 1298 A>C for the MTHFR gene (kit Elucigene CF-EU2v1, Elucigene Diagnostics); and AZFa/b/c microdeletions on the Y chromosome (Multiplex oligo-azoospermia kit-FL. Experteam). Because the cystic fibrosis conductance regulator gene (CFTR) was recently reported to have an independent role in the gene regulation in Sertoli cells (22), in the genetic assessment of HSECs we included mutations of the CFTR; CFTR F508del, CFTR F508del heterozygosis, CFTR 5T/7 T, CFTR 7 T/7 T, and CFTR poly 7 T/9 T (kit Elucigene CF-EU2v1, Elucigene Diagnostics).

HSECs and Quantitative PCR

At passage 4, 40,000 HSECs were stimulated for 72 hours with pure all transretinal (Sigma-Aldrich) or with the pan-retinoic acid receptor antagonist (23) BMS439 (Tocris Bioscience), in duplicate. Next, RNA was extracted using the ReliaPrepTM RNA Cell Miniprep System (Promega Italia Srl) and retrotranscripted with a High Capacity RNA-to-cDNA Kit. Quantitative real-time polymerase chain reaction (qPCR) was performed on the ABI PRISM 7000 thermal cycler (Applied Biosystems Italia) using the TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for RAR-beta (RARβ, probe Hs00977140_m1), laminin-4 (LAMA4, probe Hs00935293_m1), laminin-5 (LAMA5, probe Hs00966585_m1), heparan sulfate proteoglycan-2 (HSPG2, probe Hs01078536), nidogen-2 (NID2, probe Hs00201233_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, probe Hs99999905_m1; Applied Biosystems Italia). The qPCR protocol was the following: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. The level of gene expression was normalized against the housekeeping gene GAPDH, which was not modulated by transretinal stimulation (DataAssist software, ThermoFisher Scientific). All samples were run in duplicate, and the relative gene expression calculated as $2^{-\Delta Ct}$ was expressed as fold change versus vehicle (dimethyl sulfoxide) stimulated cells.

Statistical Analyses

The 105 ECM proteins, which were selected upon comparison with the Total Human Matrisome database, were submitted to Student's *t*-test statistical analysis with $P<.005$ and subsequent hierarchical clustering implemented in MeV software (v. 4.9.0). Networks for protein-protein associations by means of STRING v10 were set up with a confidence score of >0.5. Gene ontology enrichment analysis was performed to reveal upstream regulators by Ingenuity Pathway Analysis. For all other statistical tests and enrichments, $P<.05$ was considered statistically significant, and two-tailed tests were used. Continuous variables were expressed as medians and interquartile ranges.

RESULTS

Antimüllerian (AMH) Levels Were Higher in SRneg Men

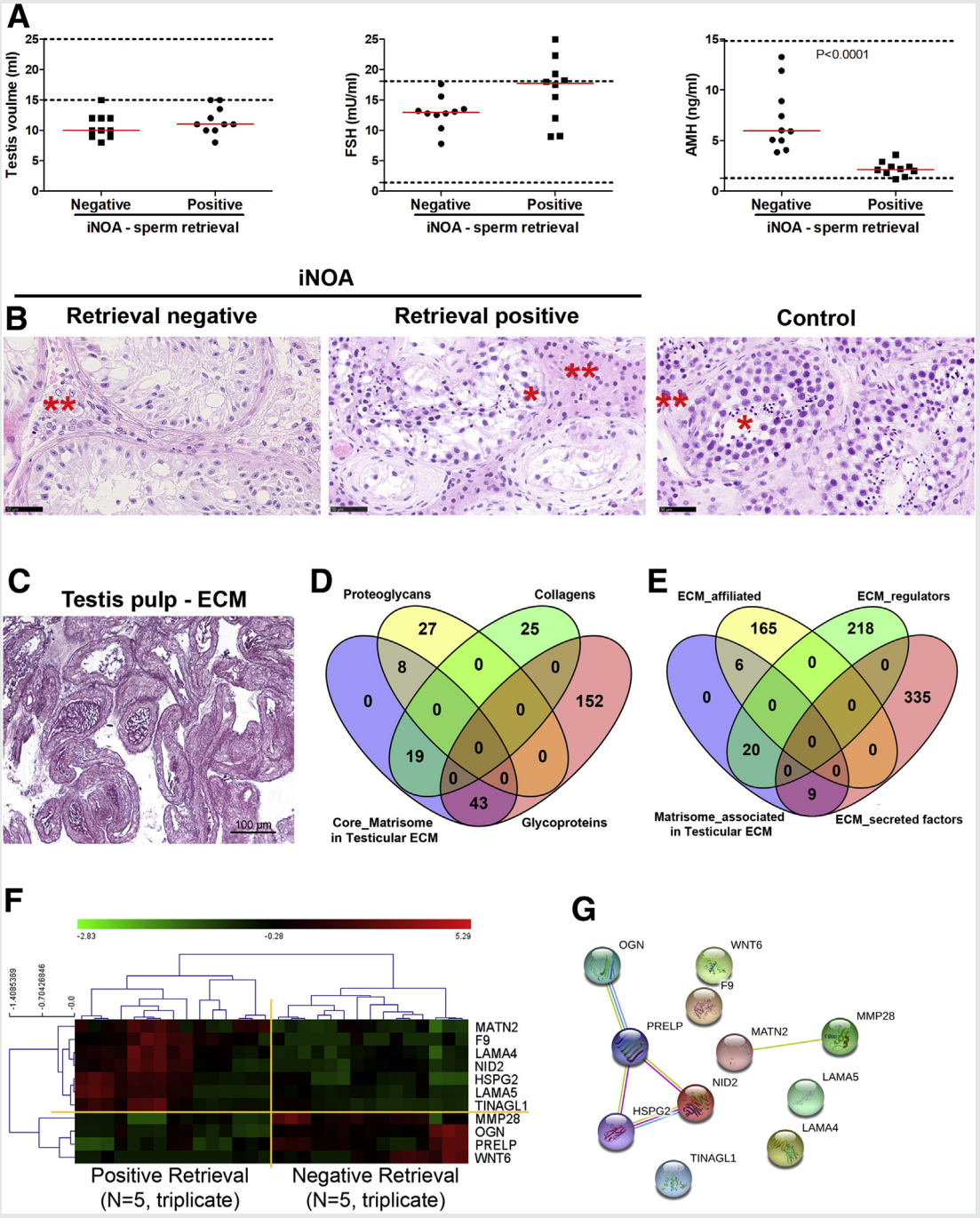
SRpos samples contained a median value of 0.038 sperm/high power field (interquartile range = 0.0033–0.48). SRpos versus SRneg cohorts were comparable for clinical and hormone variables (Supplemental Table 1); in both iNOA groups, hormone values were suggestive of primary hypogonadism, but SRneg men had higher AMH levels (Fig. 1A).

Aberrant ECM Composition of the Testis Parenchyma in SRneg versus SRpos Specimens

At histology, SRneg showed that all seminiferous tubules lacked germ cells and were lined by Sertoli cells only, thus configuring the diagnosis of SCOS and germ cell aplasia (Fig. 1B); SRpos specimens revealed a more heterogeneous pattern, characterized by the copresence of seminiferous tubules with some foci of residual spermatogenesis and tubules with hypospermatogenesis, germ cell arrest, and SCOS (Fig. 1B).

A full characterization of the biochemical composition of the ECM isolated from the frozen testicular specimens (Fig. 1C) was performed for 10 iNOA individuals (five SRpos and five SRneg specimens). Quantitative label-free proteomics analysis allowed the identification and quantification of 837 nonredundant proteins in both ECM types (mass spectrometry data are available via ProteomeXchange with identifier PXD011817): 105 were ascribed to the human matrisome (18) (Supplemental Spreadsheet), of which 70 were ascribed to the core matrisome (Fig. 1D and

FIGURE 1



Higher systemic level of AMH and aberrant ECM composition of the testis parenchyma in negative versus positive sperm retrieval specimens. (**A**) Testis volume (reported as average of the two gonads for each patient) and serum levels of FSH and AMH were evaluated in 10 iNOA men with positive sperm retrieval and 10 iNOA men with negative sperm retrieval at microTESE; dot plots depict values from the 20 iNOA men, the red horizontal bar details median values, and the dashed lines represent the range of reference values. Statistical significance was evaluated by means of two-tail nonparametric *t*-test (Mann-Whitney test). (**B**) Testis specimens from 20 iNOA men and five men with normal maturation of the germ line were histologically classified after hematoxylin-eosin staining in FFPE sections (one representative picture for each cohort). Only SCOS tubules were present in SRneg samples (Sertoli cells but no germ cells); in SRpos samples a mix of SCOS tubules and tubules with germ cells was present. The tubules of control tissue all showed normal maturation of the germ line. Scale bar, 50 μm. The red asterisk (*) represents a seminiferous tubule with germ cells and spermatozoa; the red double asterisk (**) represents Leydig cells. (**C**) The ECM was isolated from the testis tissue of five retrieval positive and five retrieval negative iNOA men for whom OCT frozen tissue was available (one representative testis-derived ECM, free of cells, is shown; scale bar, 100 μm. (**D, E**) The comparison with the matrisome database is reported. (**F**) Unsupervised hierarchical cluster analysis of label-free quantification intensities is depicted ($P<.005$, Student's *t*-test). (**G**) Networks for protein-protein associations were assessed by means of STRING v10, using a confidence score >0.55. (**G**) Networks for iNOA men were assessed by uploading the 11 proteins resulting from our proteomic analysis.

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Supplemental Table 2 and 35 to ECM-associated components (Fig. 1E and Supplemental Table 3).

Unsupervised hierarchical clustering of the 105 ECM proteins revealed significant down-regulation of seven ECM proteins and up-regulation of four proteins in the Rneg samples: a down-regulation of the major components of the basement membrane (laminin-4A [LAMA4], laminin-5A [LAMA5], nidogen-2 [NID2], and heparan sulfate proteoglycan-2 [HSPG2]) along with up-regulation of small leucine-rich proteoglycans mimecan (OGN) and prolargin (Prelp; Fig. 1F). Networks for potential protein-protein associations among the 11 deregulated ECM proteins revealed that six proteins were associated with two pathways; conversely, the remaining five proteins were not associated with each other (Fig. 1G). Gene ontology enrichment analysis based on the 11 deregulated ECM proteins was performed to reveal upstream regulators by Ingenuity Pathway Analysis; five top upstream regulators were revealed, such as vitamin K epoxide reductase complex subunit 1 (VKORC1), vitamin K-dependent gamma-carboxylase (GGCX), retinoic acid (RA), p53, and mir-663 (all P values $<5*10^{-4}$). Validation of the vitamin K and RA pathways was performed.

Decreased Signaling of Vitamin K in the Leydig Cells Was Associated with Germ Cell Aplasia

We then performed validation of VKORC1 expression by immunohistochemistry. In SRneg tissues, VKORC1 expression was present in Sertoli cells with a cytoplasmic pattern of distribution, but not in Leydig cells. Conversely, VKORC1 was expressed at similar levels in the cytoplasm of both Sertoli and Leydig cells in SRpos tissues and in the normal spermatogenesis controls (Fig. 2A).

As VKORC1 is an upstream regulator of the vitamin K–dependent coagulation factor IX (F-IX), we also tested its expression to validate our findings. Likewise, F-IX expression was detected in Sertoli cells, and at low levels in Leydig cells from SRneg individuals. Conversely, in both SRpos and controls, F-IX was observed mainly in the cytoplasm of Leydig cells (Fig. 2B). Specificity of F-IX staining was assessed in the same tissue sections by showing the absence of signal in the stromal and immune cells (Fig. 2B, inset). Staining with the Leydig cell–specific marker calretinin was performed to confirm the presence of the Leydig cells in all samples (Fig. 2C). Normal Leydig cells were detected in 14 out of 20 (70%) testicular pulps, while in the remaining six specimens Leydig cell hyperplasia was found, with no different distribution between the study groups. Moreover, no Leydig cell tumors were observed.

To show the absence of systemic deficiency of vitamin K, the peripheral prothrombin ratio was measured and found to be similar in the study cohorts and within the normal reference range (Fig. 2D).

Increased RA Signaling in Sertoli Cells Was Associated with Germ Cell Aplasia

To assess the possible contribution of RA signaling in the observed phenotypes, we analyzed the distribution of RA receptor-alpha (RARα). As the impaired AMH expression between SRpos and SRneg suggested different functionality of Sertoli cells, we focused our attention on the Sertoli cells. The nuclear localization of RARα was used to determine whether the different in vivo activation status of the RA pathway in Sertoli cells was associated with either SCOS (Fig. 3A) or the presence of sperm in the tubules (Fig. 3B). The number of Sertoli cells expressing nuclear RARα was higher in SCOS tubules associated with SRneg, compared with the counterpart of SCOS tubules of SRpos tissues. In tubules of SRpos the number of Sertoli cells with activated RARα was similar to that observed in the normozoospermic tissue (Fig. 3C). Peripheral levels of retinol fell within the reference range and were comparable in the study groups (Fig. 3D).

RA Inhibited the Expression of HSPG2 and NID2, but Not Laminins, from Sertoli Cells

RA was validated as the second upstream regulator of the composition of testicular ECM, which was identified through the gene ontology enrichment analysis. Quantitative reverse transcriptase PCR was applied to assess the role of RA for the expression of ECM proteins of the basement membrane, using primary Sertoli cells from two adult Caucasian men with normal karyotype (Fig. 4A) and without the genetic abnormalities previously associated with azoospermia (i.e., mutations of the CFTR and MTHFR gene and Y chromosome microdeletions). Phenotypic validation of Sertoli cells was performed by testing the expression of SOX9, GATA4, claudin 11, and the AR (Fig. 4B). Stimulation of Sertoli cells with exogenous all-trans RA upregulated the expression of RA receptor-beta (RARβ), HSPG2, and NID2, but not of LAMA4 and LAMA5 (Fig. 4C), also indicating specificity and lack of toxicity of the treatments.

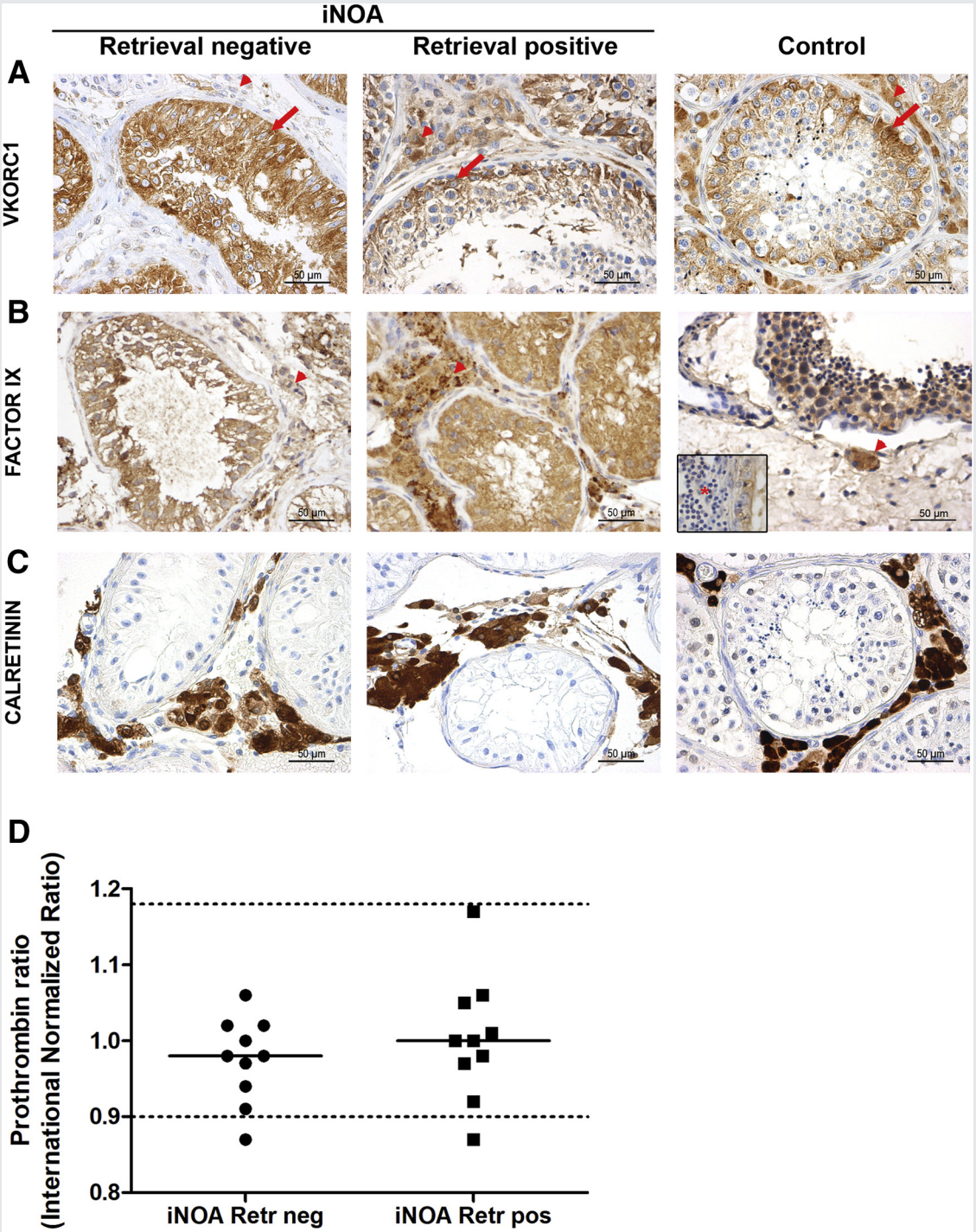
To determine whether the effect observed was directly mediated by RA signaling, we treated Sertoli cells with the pan-RA receptor inhibitor BMS493, which caused upregulation of HSPG2 and NID2 expression, but not of LAMA4 and LAMA5 (Fig. 4C), thus suggesting that retinoids are, at least partly, responsible for the deregulation of ECM molecules. Furthermore, these findings were in agreement with the gene enrichment analysis showing HSPG2 and NID2 in the same pathway, but not LAMA4 and LAMA5 (Fig. 1F).

DISCUSSION

In primary iNOA a different biochemical ECM composition of the testicular microenvironment was associated with different sperm retrieval outcomes at microTESE. Likewise, an unbalanced signaling of vitamin K and RA in germ cell aplasia was associated with an impaired ECM composition of the interstitial parenchyma and basement membrane of the seminiferous tubules.

The proteome of the human testis has recently been described (6, 7), but none of the published studies focused on the role ECM plays in testicular microenvironment homeostasis and sperm production. Here we provided the first description of the deregulation of 11 ECM proteins in men with germ cell aplasia, including components of the

FIGURE 2



Decreased signaling of vitamin K in the Leydig cells was associated with negative sperm retrieval. The expression of (**A**) VKORC1 and (**B**) coagulation F-IX was evaluated in (**C**) tissue areas positive for the Leydig cell marker calretinin, in the testis specimen from 10 iNOA men negative for sperm retrieval, 10 iNOA men positive for sperm retrieval, and five men with normal maturation of the germ line. In panels **A**–**C**, one representative picture from each cohort is shown (scale bar, 50 μm). The inset in panel B represents a negative control, showing no F-IX staining in the inflammatory infiltrate of an area of control tissue. The red arrow indicates a representative Sertoli cell; the red arrow head indicates a representative Leydig cell or cluster of Leydig cells; the red asterisk (*) indicates representative infiltrated immune cells negative for F-IX. (**D**) The vitamin K–dependent prothrombin ratio was evaluated in the peripheral serum of the 10 iNOA men negative for sperm retrieval and 10 iNOA men positive for sperm retrieval; the prothrombin ratio is expressed as an international normalized ratio, and the median value is represented by the solid bar. Statistical significance was evaluated by means of two-tail nonparametric *t*-tests.

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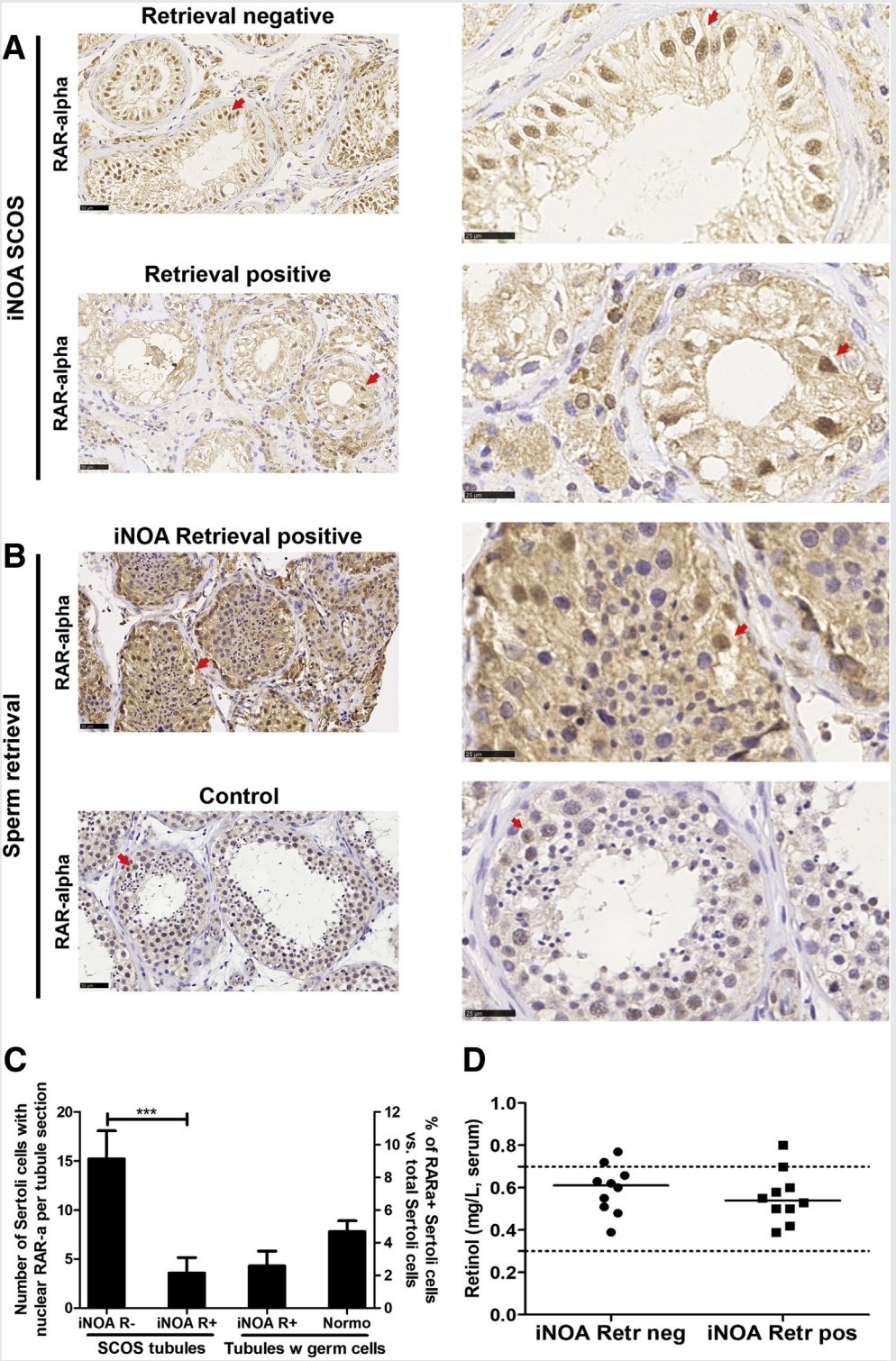
FIGURE 3



Increased signaling of RA in the Sertoli cells was associated with negative sperm retrieval. RA signaling was evaluated in the testis specimen from 10 iNOA men negative for sperm retrieval, 10 iNOA men positive for sperm retrieval, and five men with normal maturation of the germ line, focusing on the nuclear localization of RARα in the Sertoli cells of (**A**) SCOS tubules of SRneg and SRpos iNOA men, and (**B**) seminiferous tubules with sperm from the testis parenchyma of retrieval positive iNOA men and control tissue. In panels A and B one representative image for each cohort is shown; the red arrow indicates a representative Sertoli cell with nuclear localization of RARα; scale bar, 50 μm for the left panels and 25 μm for right panels. (**C**) Statistical significance by analysis of variance test followed by Bonferroni post-test correction, with data expressed as absolute number and percentage. (**D**) Retinol levels in the peripheral serum were evaluated in the 20 iNOA men stratified according to sperm retrieval; the thick lines represent the median values, and the dotted lines show the reference values; statistical significance was evaluated by means of two-tail nonparametric *t*-tests.

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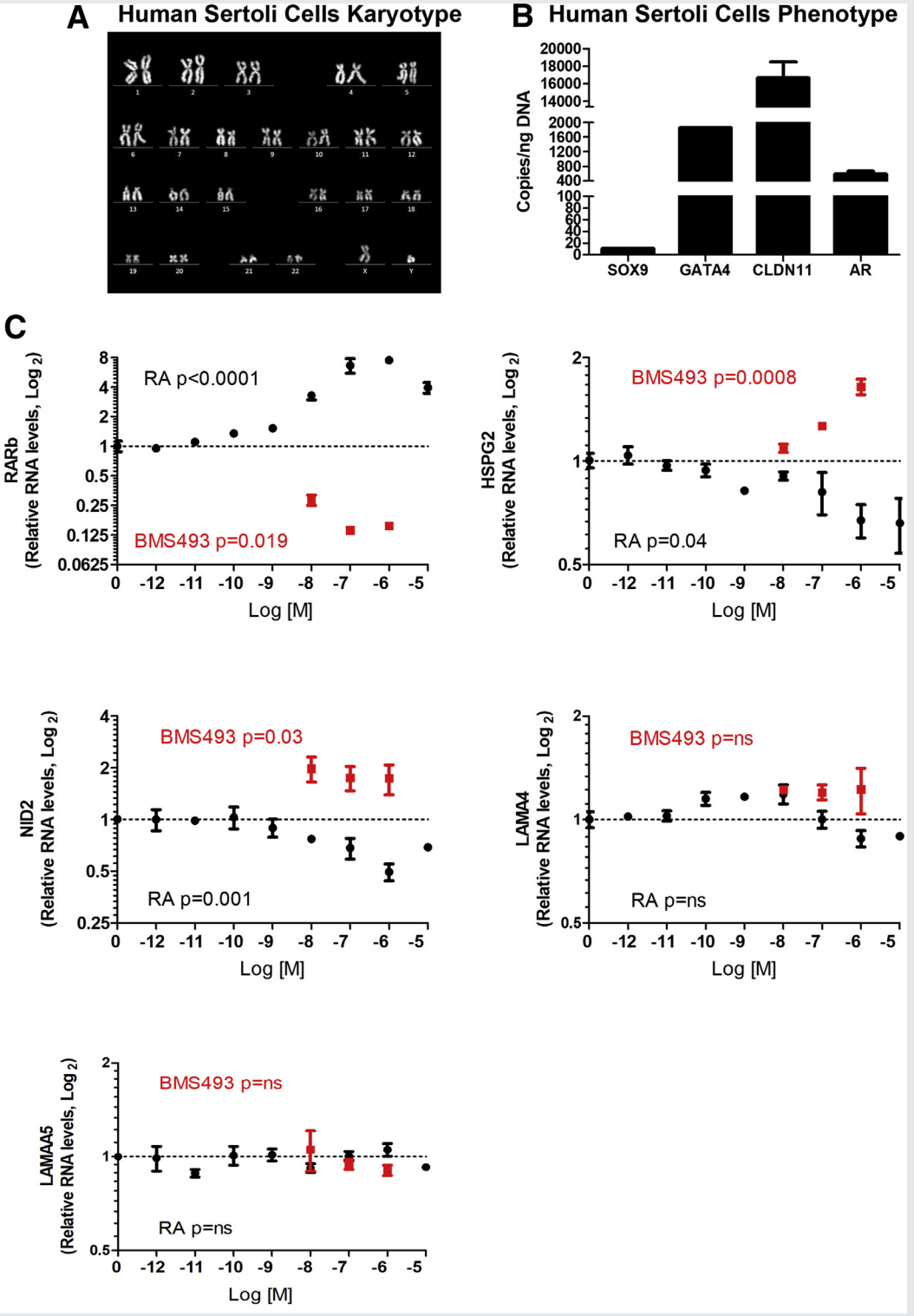
FIGURE 4



RA inhibits the expression of HSPG2 and NID-2 from Sertoli cells. Two primary Sertoli cell lines with (**A**) normal karyotipe (one representative analysis), and (**B**) normal phenotype were used to establish (**C**) dose-dependent modulation of the level of expression of basal membrane ECM RNAs by pure all transretinal (RA) and BMS493. Relative RNA levels are expressed as 2ˆΔΔCt versus GADPH as reference gene and normalized for values measured in the vehicle- (dimethyl sulfoxide–) treated Sertoli cells (represented by the dashed line). Two experiments in duplicate for each of the two primary cell lines were performed, and RARβ was used as a positive control for RA and BMS493 stimulation. Data are shown as mean ± SEM, and statistical significance was determined by two-tail analysis of variance test. SOX9 = sex-determining region Y-box 9 protein; GATA4 = GATA binding protein 4; CLDN11 = claudin 11.

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basement membrane such as LAMA4, LAMA5, NID2, and HSPG2. The down-regulation of these major components of the basement membrane was coupled with a concomitant up-regulation of OGN and Prelp, proteins that play key roles in anchoring the basement membranes to the underlying connective tissue (24, 25).

ECM is of paramount importance in the stem cell niche, where ECM receptors allow stem cells to anchor to the local microenvironment (26). At the level of the basal membrane of the seminiferous tubules, several integrins mediate the attachment of spermatogonial stem cells (27) to laminins (28, 29), and the integrin-mediated anchoring is a necessary signal for self-renewal, proliferation, differentiation, and survival of spermatogonia (30). Taken together, these findings suggest that germ cells of iNOA individuals are unable to bind to the basement membrane, which is a prerequisite for the preservation and maintenance of staminality. Overall, the deregulated expression of these components could represent one of the pathophysiological insults that eventually lead to germ cell aplasia.

We confirmed previous findings that higher circulating AMH levels were associated with SRneg outcomes at micro-TESE (13). In animal models, ARs are required for the expression of LAMA5 in Sertoli cells (31). Moreover, the expression of LAMA5 was inversely associated with the expression of AMH (32–34). Thus, the presence of LAMA5 in the basement membrane of the seminiferous tubules was described as a marker of testis differentiation, being expressed when AMH levels were low, as after the pubertal stage. In this context, AMH production is under the control of FSH up to the pubertal stage (35), and its expression is drastically reduced by testosterone (T) after puberty (36). Our findings suggest that both high serum AMH levels and low levels of LAMA5 expression in the testis with germ cell aplasia may represent an undifferentiated status of the testis, and more specifically of Sertoli cells, possibly as a consequence of a defective androgen response. Previous reports indicated that the testes of elderly men are characterized by a jeopardized spermatogenesis, with some seminiferous tubules showing foci of spermatogenesis, whereas the adjacent ones are completely without spermatogenesis and with tissue modifications including fibrosis of the wall of the seminiferous tubules themselves (37, 38). Overall, the latter findings would suggest that after testis differentiation the tissue passes through a degenerative process. These features are shared by the testis of young infertile males (37, 38). Although these findings are in contrast with the hypothesis of an undifferentiated testis in iNOA men, further studies need to test the occurrence of an earlier ECM modification in the testis of infertile men that either precedes or blocks a process of testis differentiation. This interpretation is in agreement with the so-called early aging process that would seem to clinically characterize infertile men, in particular the iNOA males who experience the occurrence of metabolic diseases and neoplasia even up to 20 years earlier than their fertile counterparts (39).

Vitamin K and vitamin A signaling were identified as upstream regulators of the ECM composition. Vitamin K was shown to sustain T production by inducing the expression of Cyp11a, regardless of LH systemic levels and its gamma-carboxylation activity (40, 41). While we did not observe any systemic difference in serum T and prothrombin ratio between groups, we showed that germ cell aplasia was associated with a decreased expression of the enzyme VKORC1 and of F-IX in Leydig cells. Altogether, these data suggested that a locally impaired signaling of vitamin K is present only in the Leydig cells of germ cell aplasia men.

Different from most mammalian cells, where RA receptors (RARs) are nuclear regardless of their cognate ligands, in Sertoli cells RARs move from the cytoplasm to the nucleus only when RA is bound (42). Thus, to establish the in vivo response of Sertoli cells to RA, the number of Sertoli cells with nuclear expression of the RARα was counted, comparing the number of RARα+ Sertoli cells in SCOS tubules of SRpos and SRneg iNOA men to that in the seminiferous tubules still containing germ cells. The greatest number of Sertoli cells with nuclear RARα was found in the SCOS tubules of Rneg, thus supporting the overactivity of RA signaling in this group. In SRpos samples, where SCOS tubules coexist with tubules with residual germ cells, the number of Sertoli cells that responded to RA was low and similar in both types of tubules, and comparable to controls as well. In addition, we reported that RA down-regulates the expression of HSPG2 and NID2 from primary Sertoli cells, whereas it does not modulate the expression of LAMA4 and LAMA5. These observations suggest a complex network of reciprocal connections, where multiple pathways interact for the dysregulation of ECM composition, both within the same cell population (e.g., Sertoli cells) and between different cell types, including the peritubular myoid cells, which are known to express laminins, type IV collagen, and fibronectin (43, 44). Systemic levels of retinol were found to be normal and similar in SRpos and SRneg cohorts, indicating no defect in the mobilization and blood circulation of the RA precursor.

Overall, these findings indicate the occurrence of different mechanisms of RA signaling in SRpos and SRneg iNOA men, and an impaired signaling of RA by Sertoli cells associated with a deregulated biochemical composition of the basal membrane of the seminiferous tubules.

Previous observations have suggested that RA inhibits FSH signaling in Sertoli cells in rodents (45), thus preventing the secretion of a variety of FSH-induced products (46) and the expression of ARs (47). This scenario would fit with our findings applied to germ cells aplasia, a condition where the high number of Sertoli cells responsive to RA might quench FSH signaling and thus the expression of the AR required for maintaining low levels of AMH. In this context, RA not only plays an important role in steroidogenesis, but it also directly affects spermatogonia proliferation and the initiation of meiosis in the adult gonads (48–53), while favoring the blood-testis-barrier formation (54). Paradoxically, either a deficit or an excess of RA resulted in testicular lesions and spermatogenetic disorders in adult animals (46). Studies in mice have revealed that during the embryonic development of Sertoli cell precursors, sequestration and degradation of RA, via expression of the degrading enzyme *Cyp26b1*, was required for the formation of prospermatogonia (55). In

agreement, *Cyp26b1* knockout mice have been shown to have increased RA levels in the embryonic testis, which is associated with the absence of germ cells and a lower testis volume in neonates (56). Accordingly, treatment of human fetal testis with RA reduced the number and proliferation of germ cells and led to an impaired organization of Sertoli cells in the seminiferous cord (57). Therefore, RA participates in the development of fetal testis, while excessive stimulation during fetal development contributes to testicular dysgenesis syndrome, thus impairing fertility in the adult age. The findings of the present study are in line with studies using preclinical animal models and human fetal testes, which showed that excessive local stimulation with RA during fetal development contributes to the development of hypogonadism and germ cell aplasia.

The study is not devoid of limitations. First, the control tissue from seminoma patients may display some degree of testicular dysgenesis. Although it is not easy to find another source of normal tissue, if not from cancer patients, great care was paid to get tissue samples from the most distant area from the tumor to avoid at best the influence of the neoplasia on the tissue, and histology confirmed normal spermatogenesis. Moreover, further studies are needed to better clarify the potential contribution of the peritubular myoid cells in the deregulation of the testicular iNOA microenvironment. Last, our study demonstrated an association between germ cell aplasia and local deregulation of vitamin A and K metabolism, but further investigations are needed to define the casual link.

Conclusions

This study demonstrates that idiopathic human germ cell aplasia is associated with increased RA signaling in Sertoli cells and a decreased signaling of vitamin K in Leydig cells and that a deregulation of these pathways contributes to the modified composition of the basal membrane of the seminiferous tubules and of the testicular ECM, occurring in the absence of impaired systemic levels of vitamin K and RA. Overall, our findings suggest that the testis of iNOA men with germ cell aplasia may be representative of a prepubertal stage characterized by an excessive response of Sertoli cells to RA, which eventually contributes to an impaired biochemical composition of the basal membrane, along with high levels of AMH, hypogonadism, and germ cell aplasia.

Given that the impaired signaling of the two vitamins was a local phenomenon, and localized in cellular populations under strict hormone control, our findings pave the way to search for local dysregulating mechanisms of the metabolic cycle of vitamin A and vitamin K, such as epigenetics silencing, post-transcriptional modifications, and a detrimental effect of the commensal microbiome (20), which may eventually shed light on the most severe male factor infertility.

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La insuficiente señalización testicular de la vitamina A y la vitamina K contribuye a la composición aberrante de la matriz extracelular en la aplasia idiopática de células germinales

Objetivo: estudiar las características patógenas del microambiente testicular somático asociado a la aplasia idiopática de células germinales.

Diseño: Estudio transversal.

Paciente (s): muestras testiculares de hombres con azoospermia no obstructiva idiopática (ANOI) prospectivamente sometidos a microdisección testicular para extracción de espermatozoides.
De las 20 muestras utilizadas para histología, 10 también estaban disponibles para el análisis proteómico. También se utilizaron células de Sertoli primarias con cariotipo y fenotipo normal.

Intervención (es): los pacientes con ANOI se dividieron de acuerdo con la recuperación de espermatozoides positiva frente a negativa en la extracción testicular de espermatozoides por microdisección, y en la matriz extracelular aislada (MEC) se realizó el análisis proteómico.

Principales medidas de resultado: Análisis proteómico de la MEC de muestras testiculares con recuperación de espermatozoides positiva y negativa. Se utilizó el enriquecimiento de ontología génica para identificar reguladores en sentido ascendente basados en las 11 proteínas de la MEC desreguladas, que se validaron por inmunohistoquímica y reacción en cadena de la polimerasa cuantitativa. Las variables continuas se expresaron como medianas y rangos intercuartiles.

Resultado (s): La aplasia de células germinales se caracterizó por un aumento de la señalización del ácido retinoico en las células de Sertoli y se asoció con una disminución de la expresión de los marcadores de membrana basal nidogen-2, heparan sulfate y proteoglycan-2. En cambio se relacionaron una disminución de los niveles del matrisoma intersticial asociado a factor IX y su regulador VKORC1. con la disminución de la señalización de la vitamina K en las células de Leydig. También se encontró alterada la expresión de otras ocho proteínas de la MEC, incluidas la laminina-4 y la laminina-5. Los niveles periféricos de las dos vitaminas estaban dentro del rango de referencia en las dos cohortes de hombres con ANOI.

Conclusión (s): identificamos la firma patogénica del microambiente somático testicular humano, proporcionando los mecanismos asociados a dos vitaminas relacionados con los determinantes moleculares de la aplasia idiopática de células germinales.