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Catarina Pedrosa Martins da Costa

Emerging Genetic Alterations Linked to Male Infertility: X-Chromosome Copy Number Variation and Spermatogenesis Regulatory Genes' Expression

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Emerging Genetic Alterations Linked to Male Infertility: X-Chromosome Copy Number Variation and Spermatogenesis Regulatory Genes' Expression

ORIENTADOR

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DEDICATÓRIA

Aos meus pais e Carolina.

1 **Type of Article: Original Research Article**

2

3 **Title: Emerging genetic alterations linked to male infertility: X-chromosome Copy**

4 **Number Variation and Spermatogenesis regulatory genes' expression**

5

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20 **Title: Emerging genetic alterations linked to male infertility: X-chromosome Copy**

21 **Number Variation and Spermatogenesis regulatory genes' expression**

22 **Abstract**

23 The etiopathogenesis of primary testicular failure remains undefined in 50% of cases.
24 Most of these idiopathic cases probably result from genetic mutations/anomalies. Novel
25 causes, like Copy Number Variation and gene expression profile, are being explored
26 thanks to recent advances in the field of genetics. Our aim was to study Copy Number
27 Variation (CNV) 67, a patient-specific CNV related to spermatogenic anomaly and
28 evaluate the expression of regulatory genes *AKAP4,* responsible for sperm fibrous sheet
29 assembly, and *STAG3,* essential for sister chromatid cohesion during meiosis. One
30 hundred infertile men were tested for CNV67 with quantitative PCR (qPCR). Quantitative
31 real-time PCR was performed to evaluate gene expression patterns of the two mentioned
32 genes in testicular biopsies from 22 idiopathic infertile patients.

33 CNV67 deletion was found in 2% of patients, with the same semen phenotype described
34 in previous studies. Expression levels of *AKAP4* and *STAG3* were downregulated in
35 infertile patients when compared to control group ($p<0.05$).

36 Resulting data reinforce the role of CNV67 in male infertility etiology. Its frequency is
37 significantly higher in oligo/azoospermic men and evidence indicates consistency of
38 phenotype. Downregulation of *AKAP4* and *STAG3* cellular transcript levels was observed
39 in the testicular biopsies, suggesting that the gene expression is altered, contributing to
40 unsuccessful sperm production.

41 As one continues to better understand about the genetics of male infertility, there will be
42 undoubtedly a shift towards better diagnosis and treatment for those patients presenting
43 idiopathic infertility.

44 **Keywords:** Male Infertility; Spermatogenesis; DNA copy number variations; Gene
45 expression.

46 **BACKGROUND**

47 An estimated 15% of couples are infertile, not achieving a clinical pregnancy after 1 year
48 of unprotected intercourse, with a great impact on the individual, couple and society [1,
49 2]. Male reproductive dysfunction is the sole or contributory cause of infertility in half of
50 the couples [3, 4], with abnormalities of sperm number (azoospermia, oligozoospermia),
51 motility (asthenozoospermia) and morphology (terathozoospermia) being frequently
52 diagnosed [5, 6].

53

54 Male infertility can be clinically divided in three main categories: acquired, congenital
55 and idiopathic, when no cause is identified [6, 7]. The idiopathic group still represents
56 50% of the cases of primary spermatogenic failure in humans [2, 8] and presently, due to
57 the lack of pathophysiological understanding, no specific treatment is offered [9]. Most
58 of the underlying causes are thought to be genetic [6, 10, 11], mainly due to
59 spermatogenesis defects [3, 12], correlated, or not, with environmental factors.
60 Spermatogenesis is a highly complex process controlled by several regulatory genes
61 which assure the correct maturation steps, from spermatogonia to sperm [3]. These men
62 are otherwise usually healthy, suggesting that any genes involved must either be only
63 expressed or be functionally required for spermatogenesis [3].

64

65 During the last years, novel tests and diagnostic tools have been employed to identify rare
66 genetic mutations and polymorphism with putative direct or indirect effects on
67 spermatogenesis. The declining cost and increased power of whole-genome sequencing
68 studies, including evaluation of the increasingly important intergenic regions of the

69 genome, is leading to nascent paths of research and likely indicate that, in the future, such
70 studies will be used on daily-basis [13]. Likewise, genetic testing of Copy Number
71 Variation and spermatogenesis's regulatory genes expression may reveal the etiology of
72 idiopathic patients and, consequently, increase the likelihood of successful paternity and
73 reduce potential risks to the progeny [13, 14].
74
75 Copy Number Variation (CNV) has raised a considerable interest among scientific and
76 medical communities. CNV is conventionally defined as a DNA segment, 1 kb or longer,
77 that is present in a variable number of copies in the genome, between individuals [15].
78 Since the first comprehensive CNV map of the human genome, in 2006, several diseases
79 have been linked to CNVs, mainly due to disruption of functional elements (either genes
80 or regulatory elements). In fact, it is well established that Y chromosome CNVs in the
81 AZF region are linked to spermatogenic impairment and are routinely analysed for
82 genetic male infertility diagnosis [9, 16]. These unbalanced quantitative variants can be
83 classified into gains (increased number of DNA copies compared to reference genome)
84 and losses (reduction or deletion compared to reference genome) [15].
85
86 Recently, high-resolution X-chromosome specific array-comparative genomic
87 hybridization (aCGH) identified CNVs which could be related with male infertility [17].
88 X chromosome genes are particularly tempting because men are hemizygous for the X-
89 genes. Since compensation by a normal allele is impossible, it is more likely that a
90 mutation may affect the fertility of an individual [6, 10]. From the reported CNVs,
91 CNV67 was one of the most promising candidates, resembling AZF deletions of the Y
92 chromosome [16, 17].
93

94 CNV67 deletion was exclusively found in infertile patients at a frequency of 1.1%
95 ($p<0.01$), ranging patient's phenotypes from azoospermia due to Sertoli-Cell-Only
96 Syndrome (SCOS) to oligozoospermia. It is localized in Xq28 and is likely to be
97 maternally inherited [16]. It has been suggested that CNV67 deletion linked to
98 spermatogenic failure may be related to highly duplicated genes of X-Cancer Testis
99 Antigen (CTA) family, the most represented X-linked testis specific family. In fact, X-
100 CTA genes comprise 10% of all X-linked genes and are expressed specifically in testis.
101 [18]. In particular, CNV67 deletion may remove the melanoma antigen family A, 9B
102 *(MAGEA9B)*, expression levels in spermatocytes and in some tumour cell lines. It may
103 also affect chromosome X open reading frame *(CXorf40A),* situated at < 1Mb from the
104 deletion and regulation elements of Heat Shock Transcription Factor Family, X-Linked
105 1/2 *(HSFX1/2)* [16, 17].

106 Gene expression profile can be used as a basis for identification of candidate genes that
107 contribute to male infertility [19-21]. To date, genetic studies in mice have identified more
108 than 200 genes that are specifically or preferentially involved in the complex regulation
109 of fertility and some are specifically expressed in the germ line [6, 10, 11, 19]. *AKAP4*
110 and *STAG3* are strong candidate genes for male infertility [7, 22].

111 The A-kinase anchor protein 4 (*AKAP4*), an X-linked member of the *AKAP* gene family,
112 encodes the most abundant protein of the spermatozoon's fibrous sheet, a cytoskeletal
113 structure surrounding the region of the principal piece of sperm flagellum [23]. *AKAP4*
114 anchors cAMP-dependent protein kinase A (PKA) to the sperm fibrous sheet, which is
115 essential for sperm capacitation, playing a central role in the regulation of normal sperm
116 motility [11, 24, 25]. In fact, studies have shown that in *AKAP4*-deficient mice, though
117 sperm count was not reduced, they were immotile, resulting in male infertility [6, 11].
118 Furthermore, another study verified no detection of *AKAP4* immunolabeling in man with

119 0% sperm mobility [19]. Therefore, *AKAP4* is likely required for the structural and
120 functional integrity of the fibrous sheath [25].
121 Stromalin 3 (*STAG3*) is a component of all meiosis-specific cohesion complexes, a large
122 ring-shaped proteinaceous structure which tethers sister chromatids, providing cohesion
123 to the structure [22, 26]. Its deletion has been related to a Premature Ovary Failure (POF).
124 Interestingly, *STAG3*-deficient male mice display a severe defect in synapses and
125 premature loss of centromeric cohesion during the early stages of prophase I, which
126 causes an arrest during the zygotene-like stage, leading to infertility [22, 27].
127
128 The aim of this study is to explore these emerging genetic alterations by quantifying the
129 copy number variation of CNV67 in a group of infertile men and consolidate the
130 pathophysiology which links CNV67 to male infertility. In addition, the expression of
131 spermatogenesis regulatory genes *AKAP4* and *STAG3* will be evaluated in infertile men
132 testicular biopsies and correlated with the (in)fertility status.
133

134 **MATERIALS AND METHODS**

135 This study includes two distinct analyses – CNV67 screening and expression profile of
136 *AKAP4* and *STAG3*. Each analysis design will be explained separately.

137 **CNV67 screening analysis**

138 *Patient samples*

139 Peripheral blood samples were collected from 100 Portuguese idiopathic infertile men,
140 with different grades of spermatogenic impairment - 44 azoospermic (AZO), 47 severe
141 oligozoospermic (SOZ), 4 oligozoospermic (OZ) and 5 normozoospermic (N) men (Table
142 1). Infertile patients were selected on the basis of a comprehensive andrological

143 examination including medical history, semen analysis, scrotal ultrasound, and hormonal
144 and genetic analysis. Patients with abnormal karyotype or Y chromosome microdeletion
145 were excluded. Normal controls were fertile normozoospermic volunteers.

146 *Genomic DNA (gDNA) extraction*

147 Peripheral blood (3–5 mL) was collected through vein puncture from all participants.
148 High molecular weight DNA was isolated using a salting out method.

149 *Quantitative PCR (qPCR)*

150 The number of copies of CNV67 on each sample was determined by Quantitative PCR
151 (qPCR). TaqMan® probes were designed by the manufacturer (Applied Biosystems,
152 Foster City, USA) and were chosen to target specific regions. Hs03323870_cn was
153 selected for the target CNV67 (labeled with FAM) and Hs03323870 was selected for
154 RNase P (labeled with VIC) and used as the reference gene. Reactions were performed in
155 triplicate in a final volume of 20 μL according to the manufacturer's instructions. Briefly,
156 the components of the reaction mix were: 4 μL genomic DNA, 10 μL 2X TaqMan®
157 Genotyping Master Mix, 1 μL 20X TaqMan® Copy Number Assay, 1 μL 20X TaqMan®
158 Copy Number Reference Assay (RNase P) and 4 μL nuclease-free water. qPCR was
159 carried out on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The
160 thermal cycling conditions were as follows: Initial enzyme activation for 10 minutes at
161 95°C, 40 cycles were performed, each one consisting of 15 seconds at 95°C and 60
162 seconds at 60°C.

163

164 *Data Analysis*

165 Applied Biosystems CopyCaller™ Software v2.0 was used to determine the copy number
166 status of each target region, and calculations were performed according to the maximum-

167 likelihood algorithm of the software. Raw copy value (RCV) represents a non-integer
168 number of copy calculated, whereas predicted copy number (PCN) is defined as an integer
169 number of copy determined by the algorithm (0, 1, 2, or 3+). As CNV67 is located on X-
170 chromosome, normal females will display PCN of 2 and normal males PCN equal to 1.
171 In the case of male alteration, Copy Number (CN) gain is defined as PCN higher than 1,
172 and PCN of 0 is regarded as CN loss.

173 **AKAP4 and STAG3 expression**

174 *Patient samples*

175 Testicular samples were collected from 22 idiopathic infertile men with AZS (used as
176 cases). Seven men with secondary infertility were used as controls. Testicular biopsies
177 were obtained to confirm the clinical diagnosis (diagnostic biopsy) or for sperm retrieval
178 (Testicular Sperm Extraction: TESE) to intracytoplasmic sperm injection (treatment
179 biopsy). Patients with abnormal karyotype or Y chromosome microdeletion were
180 excluded. Clinical information of each sample is shown in Table 2.

181 Each sample was divided into three aliquots: one was reserved for histological analysis,
182 the second (100-200mg) was processed for sperm extraction and the third (10mg) was
183 immediately transferred to a 1.5mL tube with mRNA later® solution (Ambion®, Foster
184 City, USA) and stored at -80ºC for further gene expression studies.

185

186 *RNA isolation and reverse transcription (RT) reaction*

187 After thawing the frozen pellets, cells were lysed on ice with 1000µL of TriPure Isolation
188 Reagent (Roche Diagnostics, Indianapolis, USA) and passed several times through a
189 syringe and needle. The total mRNA was then extracted according to the associated
190 protocol. At the end, RNA pellet was resuspended in 50µL of diethylpyrocarbonate

191 (DEPC)-treated RNase-free water (Promega, Wisconsin, USA) and incubated for 1h on
192 ice. RNA was then quantified in a Biotech Photometer UV 1101 (WPA, Cambridge, UK).
193 1µg of mRNA in a total volume of 10µL was reverse transcribed to complementary DNA
194 (cDNA) using qScript™ cDNA SuperMix (Quanta, Biosciences™, Gaithersburg, USA),
195 with random hexamers as the priming method and according to the manufacturer's
196 instructions.

197 *Gene expression analysis by quantitative real-time PCR (qRT-PCR)*

198 TaqMan® Gene Expression Assays were used for both targeted experimental genes
199 (*AKAP4* - Hs00275849_m1 and *STAG3* - Hs00429370_m1) All TaqMan® probes were
200 labeled with FAM dye and were purchased from Applied Biosystems. RNA 18S
201 Ribosomal (*18S*) was used as the housekeeping gene and TaqMan® Gene Expression
202 Assay was also utilized.
203 RNA expression levels were analysed by qRT-PCR on a StepOnePlus™ Real- Time PCR
204 System (Applied Biosystems). qRT-PCR was performed in a volume of 10µL, using 2µL
205 of cDNA, 2.5µL of Nuclease Free-water, 5µL of 2xKAPA probe MasterMix (Kappa
206 Biosystems, Boston, Massachusetts, USA) and 0,5µL of 20X TaqMan® Gene Expression
207 Assay for each gene, using a Fast Protocol according to manufacturer instructions.
208 Briefly, after initial enzyme activation for 2 minutes at 50°C and 20 seconds at 95°C, 40
209 cycles were performed, each one consisting of 3 seconds at 95°C and 20 seconds at 60°C.
210 Standard curves were performed with five points, in duplicates. Each PCR for relative
211 quantification was run in triplicate (technical replicates) and all genes were run together
212 with a negative control.

213 *Data analysis and statistics*

214 Data was analyzed using REST 2009 (Relative Expression Software Tool), which is a
215 standalone software tool that estimates up and downregulation for gene expression studies

216 (http://www.qiagen.com/rest). The purpose of this software is to determine whether there
217 are significant differences between samples and controls, while taking in account issues
218 of reaction efficiency and reference gene normalization. The obtained hypothesis test
219 P(H1) represents the probability of the alternate hypothesis that the difference between
220 the sample and control groups is due only to chance. Real time PCR-negativity was
221 defined by the absence of amplified product after 40 cycles and because REST software
222 uses Ct values and reaction efficiency for calculations instead of relative expressions
223 values, we proposed that the value of the last cycle of amplification (Ct = 40 cycles)
224 should correspond to the value of absence of relative expression. Wilcoxon Signed Rank
225 Test was used for the statistical analysis (StatView for Windows) with the significance
226 level set at $p < 0.05$.

227

228 **RESULTS AND DISCUSSION**

229 **CNV67 screening analysis**

230 In order to screen CNV67 deletion, 100 samples were studied from infertile men with
231 different sperm phenotypes and concentration, as previously described, by RT-PCR
232 (Table 1). Two individuals – Y3790 and Y3803 – were found to have deletion (0 copies)
233 of CNV67 (2%). Y3790 was azoospermic, diagnosed with SCOS after biopsy and Y3803
234 presented a severe oligozoospermia (2 x10^6 sperm/mL) (Fig. 1). Our data supports the
235 sperm phenotypes related to CNV67 deletion (azoospermia in a clinical context of SCOS
236 or oligozoospermia). Moreover, the findings indicate a significantly higher frequency in
237 our Portuguese population, even though our sample was quite small when compared to
238 previous studies [16, 17]. Whether the observed deletion is directly responsible for the
239 altered sperm phenotype (either affecting gene expression or regulatory elements) or is
240 related to increased genomic instability remains uncertain [17].

241 **AKAP4 and STAG3 expression**

242 Quantification of testicular mRNA levels of genes expression was carried out by qRT-
243 PCR in individuals showing spermatogenic failure. Two spermatogenesis related genes -
244 *AKAP4* and *STAG3* – were analyzed in 20 and 22 testicular biopsies samples,
245 respectively. Clinical and pathologic information on the cases and controls are presented
246 in Table 2.

247 Analysis of the qRT-PCR results was completed by using REST 2009 software. The
248 findings are summarized in Table 3. For reference gene normalization, 18S housekeeping
249 gene was used. Sample expression ratios were calculated with REST software using the
250 following formula:
251 *Relative Expression = Concentration of Gene of interest ÷ Geometric mean*
252 *(concentration of reference gene 1, concentration of reference gene 2,…)*
253 With the use of this software, the up or downregulation for each gene expression was
254 estimated comparing cases with controls. Results indicated that the *AKAP4* and *STAG3*
255 were downregulated with statistical significance ($p<0.05$) in the case group compared to
256 the control group (Table 3; Fig. 2).
257 Interestingly, 6 cases (w147, w149, w154, w176, w186, w195) did not express *AKAP4*
258 and w220 patient did not express *STAG3* (see Table 2). It has been demonstrated that the
259 reduction of gene expression in spermatogenic failure patients could not be exclusively
260 attributed to a decreased number of germ cells, but the contribution of the reduced cellular
261 expression should be also taken in account [19].
262 *AKAP4* encodes a protein involved in fibrous sheet assembly and its regulation [28], and
263 is exclusively expressed in germ cells, during the post-meiotic phase of spermatogenesis
264 [25, 29]. The findings of this study on *AKAP4* expression exhibit a statistically significant
265 difference with downregulation in the case group. Interestingly, no expression was

266 detected in several SCOS patients. However, the 2 oligo-asthenozoospermic [23] patients
267 used as controls expressed *AKAP4,* contradicting the data published in a previous study.
268 Similarly, downregulation of *STAG3* was found in the case group. The *STAG3* encodes a
269 predominant STAG protein component of cohesin complexes in primary spermatocytes,
270 participating in the telomere attachment to the nuclear periphery, telomere maintenance,
271 chromosome pairing, chromosome synapses and maintenance of sister chromatid
272 cohesion [27]. This protein is exclusively expressed in meiosis.

273

274 Gene expression profiles can be used as a basis for identification of candidate genes that
275 contribute to spermatogenic impairment. One must emphasize that an inherent problem
276 in investigating testicular expression changes is the cellular complexity of the organ [21].
277 Here we analyzed the transcriptional changes in a complete organ, with distinct germ cell
278 types. One advantage is that we revealed complex transcriptional changes related to the
279 whole testis during germ cell differentiation. The same point has the inherent
280 disadvantage, compared with isolated cell fractions, as we cannot directly identify the
281 locus of expression change. Furthermore, whether the observed differential expression
282 profiles represent the cause or consequence of spermatogenic impairment remains to be
283 elucidated. This data should be useful in delineating the patterns of gene expression
284 involved in male germline, which may contribute to understanding male infertility.

285 CONCLUSION

286 Classic male infertility tests, like karyotyping, Y chromosome microdeletions and FISH
287 analysis at somatic and germ cell levels, are no longer sufficient to investigate the
288 potential contribution of genome disorders on male infertility. A wide range of molecular

289 methods are required for better understanding of male infertility causes and, therefore,
290 increase the potential offer for a better treatment for infertile patients [30].
291 Novel genetic alterations have been identified which may be of potential clinical
292 relevance in the etiology of male infertility in the medium term, like Copy Number
293 Variation (CNV). Of all CNVs related to male infertility, X-CNV67 was one of the most
294 interesting ones [16], with a consistent phenotype and significant frequency. It is likely
295 that rare single nucleotide polymorphisms (SNPs) and CNVs, although they are rare on
296 an individual basis, collectively they can contribute to explain a significant number of
297 cases of male infertility that are currently classified as idiopathic. [13].
298
299 The present study also explores gene expression profile as an emerging genetic alteration
300 with implications in male infertility. Therefore we assessed the expression profile of
301 regulatory genes *AKAP4* and *STAG3* on infertile men testicular biopsies. Our data reports
302 altered expression of germ-line regulatory genes, providing an initial glimpse into the
303 complex regulatory network controlling germ line development. Further analyses in
304 larger series are required to better understand the biological implications of these
305 differences.
306
307 Although the importance of diagnosing genetic factors is fully recognized, the diagnostic
308 workup of infertility in men is still limited to a few genetic tests [17]. Genetic testing
309 allows clarifying an obscure infertility diagnosis and help to prevent miscarriage and
310 iatrogenic transmission of genetic defects to the offspring through Assisted Reproduction
311 Techniques (ART) [3, 4]. Therefore, we believe that efforts should be made in order to
312 identify potential genetic causes of infertility and, in this way, aid couples to make
313 informed decisions, optimize genetic testing and provide therapeutic targets [2, 31].

314

315 In conclusion, our findings merit further investigation in order to elucidate the potential
316 of CNV67 in routine fertility workup and the role of *AKAP4* and *STAG3* in male
317 infertility.

318

319 **Ethics approval and consent to participate** - The local Ethical Committees of the
320 Faculty of Medicine of University of Porto/ Centro Hospitalar S. João approved the study.
321 Informed consent was obtained from the patients before being included in this study
322 during their reproductive medical treatment.

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326

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425 **FIGURE SECTION**

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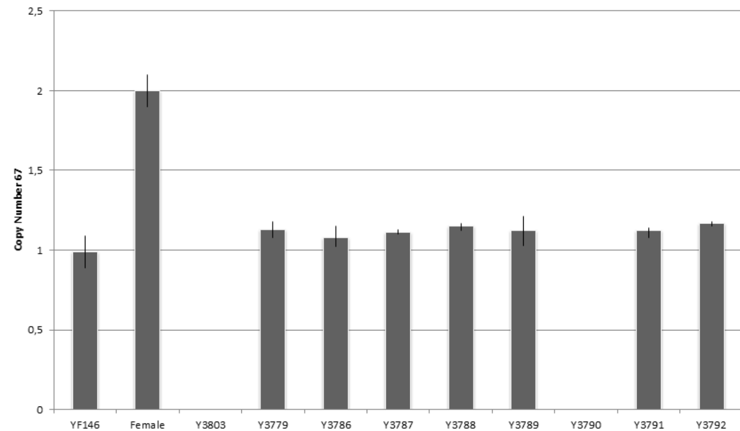

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435 Fig. 1 – Copy Number Variation 67 deletion of infertile man Y3790 and Y3803. Whiskers
436 refer to maximum and minimum of copies calculated. As CNV67 is located on X-
437 chromosome, normal females will display Predicted Number of Copies (PCN) of 2 and
438 normal males PCN equal to 1. In the case of male alteration, Copy Number (CN) gain is
439 defined as PCN higher than 1, and PCN of 0 is regarded as CN loss. YF154 – Fertile man
440 control.

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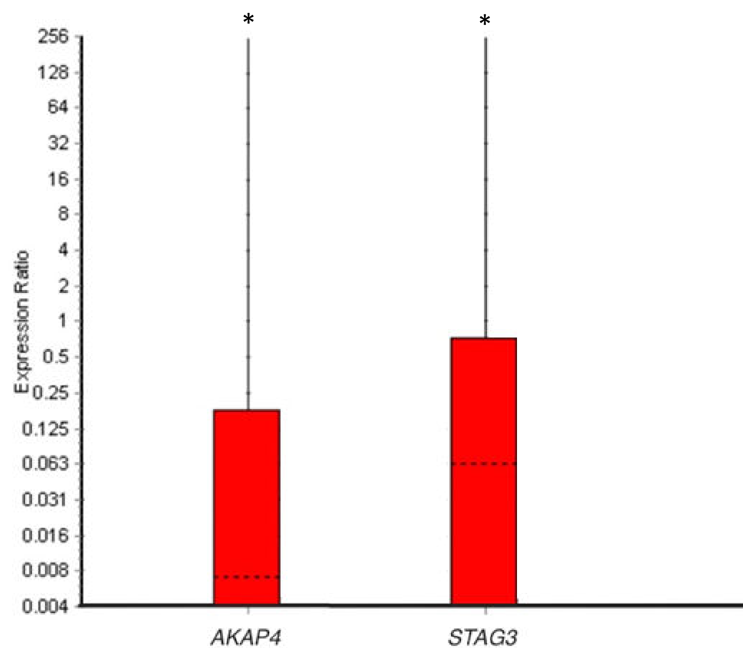

460 Fig. 2 – Expresion levels of *AKAP4* and *STAG3* in testicular samples. cDNA expression
461 was normalized using a housekeeping gene (*18S*). Boxes represents the interquartile
462 range, or the middle 50% of observations. The dotted line represents the median gene
463 expression. Whiskers represent the minimum and maximum observations. Significance
464 differences between groups are represented as: * $p<0.05$. The data was analysed by Mann-
465 Whitney-Wilcoxon test.

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Table Section

Table 1 – Clinical description of the study population

Patient's semen phenotype	(n=100)
Azoospermic	44
Severe oligozoospermic ($<5x10^6$/mL)	47
Oligozoospermic ($5\text{-}15x10^6$/mL)	4
Normal	5

Table 2 - Clinical description of the study population

Patients (n=29)	ID	Diagnosis	TESE	Clinical history
Cases (n=22)	w1	AZS	SCOS	Normal
	w77	AZS	MA	Normal
	w113	AZS	MA	Hypospadias, Orchitis (28yo, 29yo)
	w119	AZS	MA	Left testicular torsion
	w131	AZS	HP	Normal
	w140	AZS	MA	Normal
	w147	AZS	MA	Normal
	w149	AZS	SCOS	Stroke
	w152b	AZS	SCOS	Parotiditis
	w154	AZS	SCOS	Inguinal Hernia (6yo, 7yo)
	w160	AZS	HP	Normal
	w162	AZS	SCOS	Normal
	w165	AZS	HP	Normal
	w166	AZS	HP	Epilepsy
	w175	AZS	MA	Normal
	w176	AZS	SCOS	Normal
	w183	AZS	HP	Left scrotal hydrocele
	w186	AZS	SCOS	Normal
	w187	AZS	MA	Normal
	w195	AZS	SCOS	Left varicocele, Orchitis (24yo)
	w220	AZS	SCOS	Normal
	w227	AZS	HP	Hepatitis C, ex-alcoholic
Control (n=7)	w58	ANEJACUL	HP	Paraplegy
	w90	ANEJACUL	HP	Diabetes Mellitus
	w103	ANEJACUL	HP	Psycologic anejaculation
	w106	ANEJACUL	SCOS	Paraplegy
	w116	ANEJACUL	HP	Paraplegy
	w128	OLIGO-ASTHE	HP	Normal
	w164	OLIGO-ASTHE	HP	Normal

ANEJACUL – anejaculation; AZS –azoospermia; HP- hypospermatogenesis; MA –maturation arrest; OLIGO-ASTHE – oligo-asthenozoospermia SCOS – Sertoli-Cell-Only Syndrome; Shaded samples had no expression for at least one of the studied genes.

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Table 3 – *AKAP4* and *STAG3* expression results

Gene	Type	Rxn Effic.	Expression	Std. Error	95% C.I.	P(H1)	Result
18S	REF	0.9558	1.000				
AKAP4	TRG	0.9346	0.011	0.000 - 0.645	0.0-62.299	**0.007**	DOWN
STAG3	TRG	0.9829	0.050	0.001 - 2.076	0.0-114.426	**0.038**	DOWN

REF – Reference. TRG – Target. Rxn Effic. – Reaction efficiency. Std. Error – Standard Error. 95% C.I. – 95% confidence interval. P(H1) - Probability of alternative hypothesis that difference between sample and control groups is due only to chance.

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AGRADECIMENTOS

À Doutora Susana Fernandes, por toda a sua ajuda e orientação ao longo da elaboração deste trabalho, pela disponibilidade sempre demonstrada e pela forma construtiva como sempre me orientou, contribuindo para o meu enriquecimento pessoal, académico e científico.

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Aos meus amigos Ana Catarina Costa, Janete Guimarães, Maria Alves e Miguel Barbosa, que me acompanharam ao longo de todo o percurso deste trabalho com palavras de incentivo em especial nas fases de maior adversidade.

Aos meus pais, irmã e avós, por todos os bons ensinamentos que me transmitiram e me incentivaram sempre a dar o meu melhor. Agradeço todo o carinho e apoio demonstrados incondicionalmente, o que foi indispensável esta tese fosse possível. Deste modo, aqui fica um agradecimento especial.

Anexo 1

Normas da Revista “Annals of Reproductive Medicine and Treatment”

Referentes ao trabalho intitulado

“EMERGING GENETIC ALTERATIONS LINKED TO MALE INFERTILITY: X-CHROMOSOME COPY NUMBER VARIATION AND SPERMATOGENESIS REGULATORY GENES’ EXPRESSION”

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Results and Discussion: This sector should be describing the results and interpretations of the above experiments. There could be multiple subheadings or described in a single paragraph. None of the content/data should be copied.
Conclusions: This should be clearly explaining the author thoughts, highlights and limitations of the study.
Acknowledgements: Author could provide the grant details if any or express his gratitude towards his interest.
Funding Acknowledgement: Author could provide the grant details if any or express his gratitude towards his interest.
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1. **Journal Article with less than 6 authors:** Schneider LS, Dagerman KS, Insel P. Risk of death with atypical antipsychotic drug treatment for dementia: meta-analysis of randomized placebo-controlled trials. JAMA. 2005; 294: 1934-1943.
2. **Journal Article with more than 6 authors:** Rose ME, Huerbin MB, Melick J, Marion DW, Palmer AM, Schiding JK, et al. Regulation of interstitial excitatory amino acid concentrations after cortical contusion injury. Brain Res. 2002; 935: 40-46.
3. **Book Reference:** Gilstrap LC 3rd, Cunningham FG, VanDorsten JP. Operative obstetrics. 2nd edn. New York: McGraw-Hill. 2002.
4. **Chapter in a Book Reference:** Misra NC, Misra S, Chaturvedi A. Carcinoma gallbladder. In: Johnson CD, Taylor I, editors. Recent advances in surgery. London: Churchill Livingstone. 1997; 69-87.
5. **Conference proceedings:** Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference, 2001 Sep 13-15. Leeds, UK. New York: Springer, 2002.

6. **Dissertation:** Borkowski MM. Infant sleep and feeding: a telephone survey of Hispanic Americans [dissertation]. Mount Pleasant (MI): Central Michigan University. 2002.
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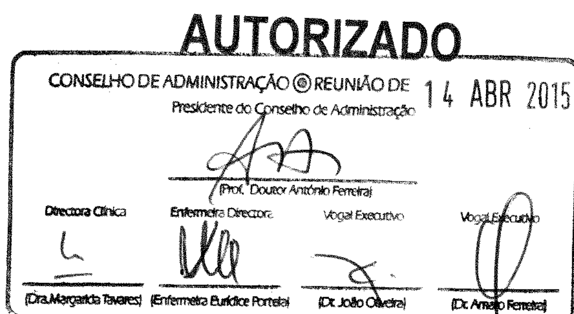
Anexo 2

Parecer da Comissão de Ética para a

Saúde do Centro Hospitalar de São João/ FMUP

CES 282-14

Direcção Clínica 26/3/15



Exmo. Senhor

Presidente do Conselho de Administração do Centro Hospitalar de S. João – EPE

Assunto: Pedido de autorização para realização de estudo/projecto de investigação

Nome do Investigador Principal: Catarina Pedrosa Martins da Costa

Título do projecto de investigação: Male infertility associated to regulatory spermatogenic genes expression

Pretendendo realizar no Departamento de Genética FMUP/Centro Hospitalar de S. João – EPE o projecto de investigação em epígrafe, solicito a V. Exa., na qualidade de Investigador, autorização para a sua efectivação.

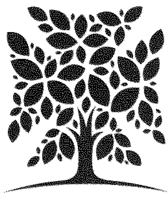
Para o efeito, anexa toda a documentação referida no dossier da Comissão de Ética do Centro Hospitalar de S. João respeitante a projectos de investigação, à qual endereçou pedido de apreciação e parecer.

Com os melhores cumprimentos.

Porto, 3 / Dezembro / 2014

O INVESTIGADOR

Catarina Costa



SÃO JOÃO

Comissão de Ética para a Saúde – Centro Hospitalar de São João / FMUP

Parecer

Título do Projecto: Male Infertility associated to Regulatory Spermatogenic Genes Expression

Nome da Investigadora Principal: Catarina Pedrosa Martins da Costa

Local onde será realizado o estudo: Departamento de Genética da FMUP, havendo autorização do respectivo Director de Serviço para a realização do mesmo.

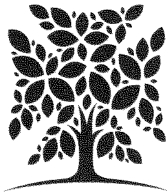
Objectivo do estudo:

Estudar a expressão de genes reguladores da espermatogénese que condicionam a infertilidade masculina. Estudar a patofisiologia que liga a Copy Nymber Variation (CNV) 67 com a infertilidade masculina.

Período previsto de conclusão: Junho 2017

Benefício/Risco: N/A

Respeito pela liberdade e autonomia do sujeito do ensaio: Prevê-se a obtenção do consentimento informado, complementado por um suporte de informação escrita para os participantes, que refere os objectivos do estudo, os riscos/benefícios, bem como a liberdade em participar.



SÃO JOÃO

Confidencialidade dos dados: está garantida a confidencialidade dos dados e esta informação será restrita aos investigadores.

A Investigadora Principal dispõe de competência técnica e científica para a realização do estudo.

Não prevê a realização de questionários.

Custos: O estudo não prevê custos acrescidos para a Instituição.

Parecer: Em face da análise do protocolo de estudo, proponho a sua aprovação pela CES da FMUP/CHSJ.

Porto, 10 de Março de 2015

O Relator

John Preto
Cirurgia Geral
35634

Dr. John Preto

7. SEGURO

a. *Este estudo/projecto de investigação prevê intervenção clínica que implique a existência de um seguro para os participantes?*

SIM ☐ (Se sim, junte, por favor, cópia da Apólice de Seguro respectiva)

NÃO ☐

NÃO APLICÁVEL ☒

8. TERMO DE RESPONSABILIDADE

Eu, Catarina Pedrosa Martins da Costa, abaixo-assinado, na qualidade de Investigador Principal, declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (com as emendas de Tóquio 1975, Veneza 1983, Hong-Kong 1989, Somerset West 1996 e Edimburgo 2000) e da Organização Mundial da Saúde, no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo no decurso do actual internamento ou da mesma consulta.

Porto, 4 de Dezembro de 2014

A Comissão de Ética para a Saúde tendo aprovado o parecer do Relator, aguarda que o Investigador/Promotor esclareça as questões nele enunciadas para que possa emitir parecer definitivo.

2014·12·16

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

Catarina Costa

O Investigador Principal

PARECER DA COMISSÃO DE ÉTICA PARA A SAÚDE DO CENTRO HOSPITALAR DE S. JOÃO

emitido na reunião plenária da CES de ___/___/___

Considerando que foram considerados satisfatórios os esclarecimentos prestados pelo investigador

A Comissão de Ética para a Saúde APROVA por unanimidade o parecer do Relator, pelo que nada tem a opôr à realização deste projecto de investigação.

2015·03·12

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética