UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Alenka HODŽIĆ

GENOMIC BIOMARKERS FOR MALE INFERTILITY

DOCTORAL DISSERTATION

Ljubljana, 2013

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GENOMSKI BIOOZNAČEVALCI PRI MOŠKI NEPLODNOSTI

DOKTORSKA DISERTACIJA

Ljubljana, 2013

This doctoral dissertation work is a completion of Interdisicplinary Doctoral Programme in Biomedicine. The work was carried out at Institute of Medical genetics and Department of Obstetrics and Gynecology, University Medical Centre Ljubljana.

On the basis of the Statute of University of Ljubljana, and by decisions of Senate of the Biotechnical Faculty and the decision of the University Senate, dated from September 9th 2011, the continuation to doctoral postgraduate studies of Interdisciplinary Docotral Programme in Biomedicine, field: Genetics, was approved. Prof. Borut Peterlin, MD, PhD as the supervisor, was confirmed.

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Date of defense: 10.7.2013

The doctoral dissertation is a result of my own research work. Undersigned I agree with publishing of my doctoral dissertation in full text on the internet page of the Digital Library of Biotechnical Faculty. I declare that the text in the electronic version is identical to the printed one.

Alenka Hodžić

KEY WORDS DOCUMENTATION

DN Dd
DC UDC 575: 616.697 (043.3)=111
CX male infertility / biomarkers / genetic / genes / genetic variability / association study / circadian rhythms / CLOCK / ARNTL / transcriptomic / gene expression / testis
AU HODŽIĆ, Alenka, B.Sc. Biology
AA PETERLIN, Borut (supervisor)
PP SI – 1000, Ljubljana, Jamnikarjeva 101
PB University of Ljubljana, Bitechnical faculty, Interdisciplinary Doctoral Programme in Bimedicine, field: Genetics
PY 2013
TI GENOMIC BIOMARKERS FOR MALE INFERTILITY
DT Doctoral Dissertation
NO IX, 77 p., 15 tab., 18 fig., 153 ref.
LA En
AL en/sl
AB Male infertility is a complex disorder and better understanding of molecular mechanisms underlying infertility may hold promise for identifying true causal factors. The main objective of this thesis was to identify genes that play a role in the etiology of idiopathic male infertility using experimental data derived from genetic association and transcriptomic studies. Based on animal models we hypothesized that genetic variability in circadian rhythm genes is associated with male infertility. On the study group consisted of 517 patients with idiopathic infertility and a control group of 444 fertile men we tested for association 8 polymorphisms in the ARNTL and CLOCK genes and found statistically significant difference in genotype distribution in the CLOCK gene: rs11932595 ($p=6\cdot10^{-5}$), rs6811520 ($p=2\cdot10^{-3}$) and rs6850524 ($p=0.01$). Further analyses of haplotypes were consistent with genotyping results. Additionally, we hypothesized that new mechanisms underlaying pathogenesis of infertility can be identified with microarray - transcriptomic approach. In 10 patients with severely impaired spermatogenesis and 10 controls, we identified more than 6000 differentially expressed genes and found enrichment of functional categories including protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis, lysosome, phagosome and Escherichia coli infection which correspond to inflammation and autoimmune activity was found. The cell type specific analysis revealed no significantly expressed genes in the Sertoli and Leydig cells between severely impaired and normal spermatogenesis patients. With meta-analysis of our and existing transcriptomic studies, we confirmed the transcriptional changes observed in our study and revealed new genes related to pathways associated with oxidation-reduction, response to organic substance, steroid biosynthetic process, response to wounding and regulation of apoptosis. In conclusion, our results provide evidence of novel association between circadian genes and male infertility and contribute to understanding of gene expression involved in pathogenesis of male infertility.

KLJUČNA DOKUMENTACIJSKA INFORMACIJA

ŠD Dd
DK UDK 575: 616.697 (043.3)=111
KG moška neplodnost / biooznačevalci / genetika / geni / genetska variabilnost / asocijacijska študija / cirkadiani ritmi / CLOCK / ARNTL / transkriptom / gensko izražanje / testis
AV HODŽIĆ, Alenka, univ. dipl. biol.
SA PETERLIN, Borut (mentor)
KZ SI – 1000, Ljubljana, Jamnikarjeva 101
ZA Univerza v Ljubljani, Bitehniška fakulteta, Interdisciplinarni doktorski študijski program Bimedicina, področje genetika
LI 2013
IN GENOMSKI BIOOZNAČEVALCI PRI MOŠKI NEPLODNOSTI
TD Doktorska disertacija
OP IX, 77 str., 15 pregl., 18 sl., 153 vir.
IJ En
JI en/sl
AI Moška neplodnost je kompleksna reproduktivna motnja pri človeku, zato razumevanje molekularnih mehanizmov ponuja možnost odkrivanja vzročnih dejavnikov za njen nastanek. Glavni cilj disertacije je bil odkriti gene, ki igrajo vlogo v etiologiji idiopatske moške neplodnosti z eksperimentalnimi podatki genetskih asociacijskih in transkriptomskih študij. Na osnovi zaključkov študij na živalskih modelih, smo sklepali, da je genetska variabilnost v genih, ki uravnavajo cirkadiane ritme, povezana z moško neplodnostjo. Na skupini 517 bolnikov z idiopatsko neplodnostjo in kontrolni skupini 444 plodnih moških, smo preverili prisotnost asociacije 8 polimorfizmov v genih ARTNL in CLOCK in odkrili statistično pomembno razliko v pogostosti genotipov CLOCK gena pri polimorfizmih rs11932595 ($p=6\cdot10^{-5}$), rs6811520 ($p=2\cdot10^{-3}$) in rs6850524 ($p=0.01$). Rezultati nadaljnjih analiz haplotipov v izbranih genih so se ujemali z rezultati genotipizacije posameznih polimorfizmov. Nadalje smo domnevali, da lahko nove mehanizme, pomembne pri nastanku moške neplodnosti, odkrijemo s preiskovanjem sprememb na nivoju transkriptoma z metodo mikromrež. Pri 10 pacientih z izrazitimi motnjami v spermatogenezi in 10 kontrolnih primerih smo identificirali več kot 6000 različno izraženih genov in pri teh ugotovili obogativev funkcijskih skupin povezanih s premeno beljakovin v endoplazemskem retikulumu, s proteolizo vodeno z ubikvitinskimi modifikacijami, s funkcijami lizosomov in fagosomov, z okužbo z Escherichio coli in z vnetjem ter avtoimunimi procesi. Celično specifična analiza transkriptomskih podatkov ni pokazala pomembnih razlik v izražanju genov v Sertolijevih in Leydigovih celicah pri bolnikih z okvarjeno in normalno spermatogenezo. Z meta-analizo naše in drugih obstoječih študij transkriptoma smo potrdili zaznane spremembe v izražanju genov in okrili nove gene, pomembne pri uravnavanju celičnega oksidacijsko-redukcijskega potenciala, pri odgovoru na organske molekule, pri biosintezi steroidov, pri odgovoru na tkivne poškodbe in pri regulaciji programirane celične smrti. Naši rezultati dajejo nove dokaze za obstoj povezave med cirkadianimi geni in moško neplodnostjo ter doprinašajo k razumevanju sprememb v izražanju genov, vpletenih v patogenezo moške neplodnosti.

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ABBREVIATIONS AND SYMBOLS

ARNTL	aryl hydrocarbon receptor nuclear translocator-like
AZO	azoospermia
CLOCK	clock circadian regulator
CFTR	cystic fibrosis transmembrane conductance regulator
CABVD	congenital bilateral absence of vas deferens
csSAM	cell type-specific significance analysis of microarrays
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
ED	ejaculatory disorders
EMA	early maturation arrest
ER	endoplasmic reticulum
FSH	follicle stimulating hormone
FDR	false-discovery rate
GH	germinal hypoplasia
GEO	Gene Expression Omnibus repository
GO	Gene Ontology terms
GWAS	Genomic Wide Associations Study
HS	hypospermatogenesis
IPA	ingenuity pathway analysis
J.S.	Jonsen score
KEEG	Kyoto Encyclopedia of Genes and Genomes
KS	Klinefelter's syndrome
LD	linkage disequilibrium
LH	Leydig cell hyperplasia
LMA	late maturation arrest
MA	maturation arrest
MDS	performed multidimensional scaling
NMF	non negative matrix factorization method
NOA	non obstructive azoospermia
OA	obstructive azoospermia
PCA	principal component analysis
PCD	primary ciliary dyskinesia
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
SCOS	Sertoly Cell Only Syndrome
SCN	suprachiasmatic nucleus
SNP	single nucleotide polymorphisms
TFBS	transcription factor binding sites
WHO	World Health Organization

1 INTRODUCTION

Infertility affects about 9 % of couples (Boivin et al., 2007) and male factors contribute to approximately half of them (Agarwal and Said, 2003). About one third of infertility cases are unexplained (idiopathic male infertility), mostly due to our poor understanding of basic molecular mechanisms underlying male fertility (Jungwirth et al., 2012; Matzuk and Lamb, 2002). It is estimated that genetic factors are implicated in the pathogenesis of 50 % of males with idiopathic infertility (Hwang et al., 2010).

Genetic factors implicated in male infertility are at every level of genetic information, from chromosomes to genes to nucleotides (O'Flynn et al., 2010). Chromosomal abnormalities, Y chromosome microdeletions, monogenic disorders (Huynh et al., 2002) and genetic variability in several genes have been associated with male infertility (Tüttelmann et al., 2007; Shen et al., 2012; Chengyong et al., 2012). Anyhow, already known genetic factors that have been linked to infertility do not explain the considered contribution of genetic factors in 50 % of males with idiopathic infertility, indicating the need for further research in this area. In the search for new genes as potential biomarkers for male infertility two approaches can be used: hypothesis based and hypothesis free approaches.

For a hypothesis-based approach, candidate genes could be selected on the basis of current understanding of pathogenic mechanisms involved in male infertility, like inflammation processes or infections (Wolff et al., 1991; Purvis and Christiansen, 1993), or based on available research in animal models (Christensen and Carrell, 2002). Studies of animal models have identified hundreds of genes that influence reproduction and have uncovered various pathogenic pathways that could affect infertility in humans (Hackstein et al., 2000; Jamsai and O'Bryan, 2011). Among other, infertility is related also with mutations in CLOCK and ARNTL genes in different animal species, which are the key genes of the circadian system pathway (Boden and Kennaway, 2006). Circadian rhythms have a major role in maintaining homeostasis and proper body function including reproductive capacity (Kennaway et al., 2012). This is highlighted most obviously in animal models. In vitro shifted light-dark cycles influenced the rhythm of sperm release in gypsy moth, showing that the testis-seminal ducts complex contains circadian pacemaker, which controls the rhythm of sperm movement in these insects (Giebultowicz et al., 1989). A mutation of circadian genes influences reproductive fitness in Drosophila melanogaster (Beaver et al., 2002). Beaver et al. (2002) have showed that clock-mutant males produce significantly fewer progeny, and release smaller quantities of sperm. Studies of ARNTL knock-out mice have shown deficiencies in steroidogenesis (Alvarez et al., 2008), namely, male ARNTL Knock Out mice had altered levels of reproductive hormones, indicating a defect in testicular Leydig cells. The circadian clock system as well as primary clock genes, CLOCK and ARNTL, are highly conserved between species (Vansteensel et al., 2008). Molecular components of the circadian clock network represent positive and negative transcriptional-translational feedback loops of many genes (Okamura et al., 2003). CLOCK and ARNTL genes represent the central node in the network, generating a positive loop, and heterodimerizing and initiating the transcription of other clock genes (Zhang et al., 2004). Resultant proteins forming the negative feedback loop inhibit the CLOCK and ARNTL transcriptional activity (Reppert and Weaver, 2002). Despite many data on the influence of the circadian system in animal reproduction, there is very limited knowledge

regarding the role of the circadian system in infertility in humans. Only a few studies have investigated the role of the circadian system in male fertility (Zhang et al., 2012; El-Helaly et al., 2010; Ortiz et al., 2010).

With regard to the hypothesis free approach, a lot of information of the etiology, pathogenesis and homeostatic mechanisms underlying infertility has been recently derived from transcriptomic, proteomic and metabolomic profiling levels (von Kopylow et al., 2010; Johnston et al., 2005; Pilch and Mann, 2006; Deepinder et al., 2007). 'Omics' technologies, enabling different perspectives, all contribute to revealing the processes underlying disease and have already demonstrated their potential. Transcriptome studies allow the simultaneous detection of the expression of almost all genes in the human genome, thereby enabling complete insight into gene regulation linked to molecular pathogenesis and homeostatic mechanisms, and gene alterations on the transcriptomal level (He et al., 2006). To date, few studies have used global expression profiling to gain clues in the idiopathic male infertility. Microarray analysis were performed on previously defined and homogeneous testicular pathologies (Fieg et. al., 2007; Spiess et al., 2007) or on samples with a range of pathological phenotypes present in patients with non obstructive azoospermia (Ellis et al., 2007; Okada et al., 2008). Although information on groups of genes associated with spermatogenesis, sperm function, mitosis and inflammation processes was acquired from these studies, results are not consistent and are difficult to interpret. Also, the main focus was on transcriptome changes in germ cells, whereby possible effect of altered expression in somatic cells have been neglected. Even though spermatogenesis represents the maturation of germ cells, it is dependent on other cell types, and somatic cells have essential functions through all phases of spermatogenesis (Wistuba et al., 2007). Thus, while in the tubules, Sertoli cells have multiple functions, for the support, protection, nutrition, proliferation and maturation of germ cells (Wilhelm et al., 2007; Griswold, 1998), whereas Leydig cells in the interstitium are required for endocrine regulation (Renhsan et al., 2009).

Therefore, we formulated two hypotheses:

1. We hypothesized that the circadian system is associated with male infertility, and furthermore, that genetic variability of the CLOCK and ARNTL genes may be associated with infertility in humans.

2. We hypothesized that with a transcriptomic study we could identify new mechanisms underlying the pathogenesis of infertility and that with additional focus on Sertoli and Leydig cells we could find novel altered genes in patients with severely impaired spermatogenesis. Also, we hypothesized that with the integration of our results with previous transcriptomic studies we could contribute to the reliability of data.

For the purpose of this research we performed a retrospective case-control genetic association study of polymorphic sites in these two genes on a population of patients with male infertility in comparison with a fertile male control population. We also performed global gene expression profiling on testis samples belonging to patients with severely impaired and normal spermatogenesis and cell type-specific analysis of microarrays to get more insight on possible molecular changes in Sertoli and Leydig cells during impaired

spermatogenesis. Furthermore, we performed meta-analyses of gene expression data across available transcriptomic studies.

We expected that our results would contribute to the identification of genetic mechanisms in the infertility and could serve as a new basic knowledge for a better understanding of the etiology of idiopathic male infertility.

2 LITERATURE REVIEW

2.1 DEFINITION OF MALE INFERTILITY

The World Health organization (WHO) defines infertility as the inability of a sexually active, non-contracepting couple to achieve spontaneous pregnancy in on year (Manual …, 2000). This definition encompasses couples who have reduced fecundability and consequently a prolonged time to pregnancy, those who are subfertile and those who will never achieve spontaneous pregnancy (Gnoth et al., 2005).

2.2 Epidemiology

Infertility occurs in about 9 % of couples worldwide (Boivin et al., 2007) and male factors contribute to approximately half of them (Agarwal and Said, 2003). Although incidence of infertility varies throughout different countries, the prevalence between more and less developed countries is similar.

Despite considerable research efforts, in about one third of infertility cases the etiology is still unknown and it is termed 'idiopathic infertility'. It is estimated that unexplained cases are mostly due to our poor understanding of basic molecular mechanisms underlying male fertility (Jungwirth et al., 2012; Matzuk and Lamb, 2002) and that genetic factors are implicated in the pathogenesis of 50 % males with idiopathic male infertility (Hwang et al., 2010).

2.3 Clinical characteristics

The initial classification of male infertility is based on the results of semen analyses. According to the WHO criteria (Laboratory …, 2000), main forms of male infertility can be classified as:

- Oligozoospermia, decreased number of spermatozoa (<15 million spermatozoa/mL)
- Asthenozoospermia, decreased sperm motility (<32 % motile spermatozoa)
- Teratozoospermia, abnormal forms of sperm (<4 % normal forms), or
- OligoAsthenoTeratozoospermia, when previously mentioned abnormalities come together.

The most severe form of male infertility is azoospermia (AZO) (Cocuzza et al., 2013). Azoospermia is defined as a complete absence of spermatozoa. The prevalence of azoospermia is approximately 1 % of all men and more than 15 % of infertile men (Oates, 2012). Azoospermia may result from a lack of spermatozoa production in the testis (non-obstructive azoospermia, NOA) or from an inability of produced spermatozoa to reach the emitted semen (obstructive azoospermia, OA) (Schlegel, 2004).

In non-obstructive azoospermia or spermatogenic failure it is necessary to differentiate between the following: reduced spermatogenesis (hypospermatogenesis), maturation arrest (MA) that can occur at an early stage of spermatogenesis (early maturation arrest, EMA) -

arrest at the spermatocyte level or at late stage of spermatogenesis (late maturation arrest, LMA) - arrest at the spermatide level, Sertoli Cell-Only Syndrome (SCOS), complete failure of spermatogenesis resulting only in Sertoli cell line in the seminiferous tubules, and Leydig cell hyperplasia (LCH), an increased number of testicular Leydig cells.

Obstructive azoospermia may result from epididymal, vassal or ejaculatory duct pathology (Baker and Sabanegh, 2013). Duct blockage can be also caused by infection. In these cases, spermatogenesis is normal even though the semen lack spermatozoa.

Uncommon, but also important in infertility are disorders of ejaculation (Hendry, 1999). One of these disorders is anejaculation. Anejaculation is ordinarily combined with central or peripheral nervous system dysfunction or with drugs.

2.4 Etiology

Multiple factors play a role in the etiology of male infertility. Infertility can be the result of varicocele, endocrine disturbances, urogenital infections, congenital abnormalities, genetic abnormalities and immunological factors (Jungwirth et al., 2012). Depending on etiology, causes of infertility can be classified into pre testicular, testicular and post testicular associated causes (Table 1) (De Kretser and Baker, 1999).

Table 1: Etiological factors in male infertility (De Kretser and Baker, 1999: 3444)
Preglednica 1: Vzročni dejavniki za moško neplodnost (De Kretser in Baker, 1999: 3444)

Pretesticular	Post-testicular	Testicular
Endocrine	Obstructive	Genetic
Hypogonadotrophic	Epididymal	Klinefelter's syndrome
Hypogonadism	Congenital	Y chromosome deletions
Coital disorders	Infective	Immotile cilia syndrome
Erectile dysfunction	Vasal	Congenital
Psychosexual	Genetic: cystic fibrosis	Cryptorchidism
Endocrine/neural/vascular	Acquired: vasectomy	Infective (orchitis)
Ejaculatory failure	Epididymal hostility	Antispermatogenic agents
Psychosexual	Epididymal	Heat
Post genitourinary surgery	asthenozoospermia	Chemotherapy
Neural	Accessory gland infection	Drugs
Drug related	Immunolgical	Irradiation
	Idiopathic	Vascular
	Post-vasectomy	Torsion
		Varicocele

2.4.1 Genetic factors in male infertility

Genetic factors are implicated in about 15-30 % of male infertility causes (O'Flynn et al., 2010). Among the most known and frequent genetic factors implicated in male infertility are chromosomal abnormalities, Y chromosome microdeletions and monogenic disorders (Huynh et al., 2002).

2.4.1.1 Chromosomal abnormalities

Chromosomal abnormalities are implicated in about 5 % of infertile men, and 15 % in the population of azoospermic males (Ferlin et al., 2007). Chromosomal abnormalities can be numerical or structural.

Numerical abnormalities include Klinefelter's syndrome (KS), the most common chromosomal disorder caused by incorrect chromosomal number. The prevalence of KS is 5 % in men with severe oligozoospermia and 10 % in azoospermic men (Foresta et al., 2005). In most of the cases KS is characterized by a 47,XXY chromosome complement, but also mosaic forms such as 47,XXY/46XY, 48XXXY, 48,XXYY and 49,XXXXY have been reported to manifest KS (Visootsak and Graham, 2006).

Numerical chromosomal abnormalities involve also men with karyotype 46,XX and men with karyotype 47,XYY (Van Assche et al., 1996).

Structural chromosomal abnormalities are more often present in men with oligozoospermia, and include Robertsonian and reciprocal translocations. Robertsonian translocations were found in 1.6 % of oligozoospermic and 0.09 % of azoospermic men (Van Assche et al., 1996). Reciprocal translocations are the cause of infertility in approximately 1.4 % men (Dong et al., 2012).

2.4.1.2 Y chromosome microdeletions

Y chromosome microdeletions are the second common genetic cause of infertility. These microdeletions are the most frequent molecular cause of non obstructive azoospermia with prevalence of 15 % (Foresta et al., 2001) and severe oligozoospermia with prevalence about 7 % (Dohle et al., 2002). It is a deletion of AZF region, located on the long arm of Y chromosome, containing essential genes for proper spermatogenesis, e.g. DAZ, USP9Y, RBMY1 in BPY2 (Sadeghi-Nejad and Oates, 2008).

2.4.1.3 Monogenic disorders

Mutations in several genes, SYCP3, CREM, FHL5, PRM1, NALP14, KLHL10, have been associated with severely impaired spermatogenesis (Miyamoto et al., 2003; Hwang et al., 2010).

About 2 % of infertile men have mutations in the Androgen receptor (AR) gene (Ferlin et al., 2006). Mutations of the AR gene can lead to androgen insensitivity syndrome and Kennedy syndrome.

Primary ciliary dyskinesia (PCD) can also affect fertility (Schwabe et al., 2008). Mutations inDNAH5 and DNAI1 genes are associated with PCD.

Mutations of the CFTR gene have been found in about 75 % of patients with congenital bilateral absence of vas deferens (CBAVD) (Dörk et al., 1997). CABVD is a form of obstructive azoospermia accounting about 1 %-2 % infertility causes (Lee et al., 2009).

2.4.2 Association studies in finding candidate genes for male infertility, hypothesis based approach

Association studies have been extensively employed to assess genetic contributions to male infertility. Until now, in relation to male infertility, genetic variability in 207 genes was studied (Yu et al., 2008). Of these 207 genes, 124 have been the subjects of only one published study, 64 of one to five and 8 genes of five to ten studies. Only 11 genes have been studied ten or more times (Figure 1).

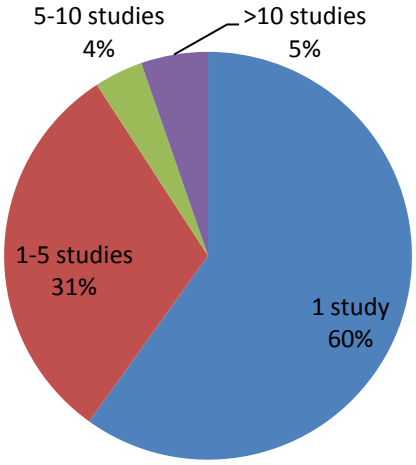

Figure 1: Currently available evidence of genes investigated in genetic association studies
The numbers presented in the figure reflect number of studies that investigated possible association of male infertility with polymorphisms in a particular gene.
Slika 1: Trenutno dostopni dokazi za gene, preiskovane v genetskih asociacijskih študijah
Številke prikazane na grafu odražajo število študij, kjer so preiskovali možno povezanost moške neplodnosti s polimorfizmi v določenem genu.

Several studies have reported positive associations of mutations and single gene variants, such as gr/gr deletions, MTHFR, POLG, DAZL, USP26, FSHR, GSTM1 null genotype and AR mutations. Meta-analyses found that only AZF gr/gr deletions, MTHFR 677C->T (Tüttelmann et al., 2007; Stouffs et al., 2011; Gupta et al., 2011; Wei et al., 2012), MTHFR 1298 A->C polymorphism (Shen et al., 2012) and GSTM1 null genotype (Chengyong et al., 2012; Tang et al., 2012) show consistent evidence of association.

Important contribution to knowledge on genetic factors playing a role in male infertility has been contributed by genome-wide association studies (GWAS). Among nine associated loci identified with the GWAS, prominent candidates for male fertility included USP8, UBD, EPSTI1 and LRRC32 genes (Kosova et al., 2012).

2.4.2.1 Genetic animal model studies

Contrary to fact that a relatively small number of genes affecting fertility is identified in humans, studies of animal models have identified hundreds of genes that influence reproduction (Christensen and Carrell, 2002). Studies in the fruitfly Drosophila

melanogaster show that more than 1500 genes contribute to male fertility (Hackstein et al., 2000). In mice, it has been estimated that over then 400 genes are essential for proper reproduction (Jamsai and O'Bryan, 2011; Cooke and Saunders, 2002). Animal studies are a significant part to our understanding of the basic biological mechanism and the effects of disruption of key processes. Studies of animal models showed that disruption of the circadian system strongly influences fertility (Kennaway et al., 2012). Circadian rhythms have a major role in maintaining homeostasis and proper body function. The testis-seminal ducts complex in gypsy moth has a circadian pacemaker which controls the rhythm of sperm movement (Giebultowicz et al., 1989). A mutation of circadian genes influences reproductive fitness in Drosophila melanogaster (Beaver et al., 2002). Beaver et al. (2002) have showed that clock-mutant males produce significantly fewer progeny and release smaller quantities of sperm, whereas the studies of ARNTL knock-out mice have shown deficiencies in steroidogenesis (Alvarez et al., 2008), namely, male ARNTL KO mice had altered levels of reproductive hormones, indicating a defect in testicular Leydig cells.

2.4.2.2 Circadian system

Circadian rhythms are daily rhythmic variations that are found in most living organisms from cyanobacteria to mammals (Delaunay and Laudet, 2002). The term *circadian* comes from the Latin *circa*, meaning "around" (or "approximately"), and *diem* or *dies*, meaning "day". Circadian rhythms synchronize a wide variety of behavioral, biochemical or physiological processes (Sukumaran et al., 2010). Any disruption of these rhythms may affect human health. Circadian rhythms are controlled by an endogenous oscillator, the circadian clock (Dibner et al., 2010). The circadian clock enables organisms to anticipate environmental changes and prepare the organism to adapt and respond to them. Circadian clocks are self-sustained and can oscillate autonomously even in the absence of environmental signal. However, they are adjusted to the local environment by external cues, which reset the system daily and prevent from free-running out of phase. The major external cue of the central clock is light. In mammals, the central core of the circadian clock, also called the 'master clock', is located in the suprachiasmatic nucleus (or nuclei) (SCN), a pair of distinct groups of cells located in the anterior part of the hypothalamus (Karatsoreos and Silver, 2007). Beside the 'master clock' circadian oscillators are also present in other parts of brain and in other organs, and are called 'peripheral clocks' (Kwon et al., 2011). The SCN recives signals from the environment and coordinates the oscillating activity of peripheral clocks so that a coherent rhythm is ensured at the organism level.

2.4.2.3 Molecular regulation

Molecular components of the circadian clock network represent positive and negative transcriptional-translational feedback loops of many genes (Okamura et al., 2003). In mammals, the core circadian clock genes are: CLOCK, ARNTL, casein kinase I ε, cryptochromes 1 and 2, Period 1, 2 and 3, and Rev-erb-α. CLOCK and ARNTL genes represent the central node in the network, generating a positive loop (Figure 2) (Beccket and Roden, 2009; Zhang et al., 2004). These proteins are members of the family of basic-helix-loop-helix transcription factors that heterodimerize via the PAS domains and induce the expression of Per and Cry genes which generate negative loop by binding to the E-box elements (CACGTG) present in the promoter of these genes. In turn, Per and Cry proteins,

after reaching critical concentration, form a heterodimeric repressor complex that translocates into nucleus to inhibit transcriptional activity of CLOCK: ARNTL complexes. The CLOCK: ARNTL complex also induces the transcription of retinoic acid-related orphan nuclear receptors, Rev-erbα and Rora. Rev-erbα and Rora subsequently compete to bind retinoic acid-related orphan receptor response elements (RREs) present in ARNTL promoter. While RREs activate the transcription of ARNTL gene, Rev-erbα repress the transcription process. The autoregulatory feedback loops take ~24 h to complete a cycle and constitute a circadian molecular clock.

These proteins are post-translationally modified by phosphorylation and ubiquitination (Reppert and Weaver, 2002; Hirota and Fukada, 2004). These processes contribute to the precision of the circadian clock by affecting the stability and nuclear translocation of clock core proteins.

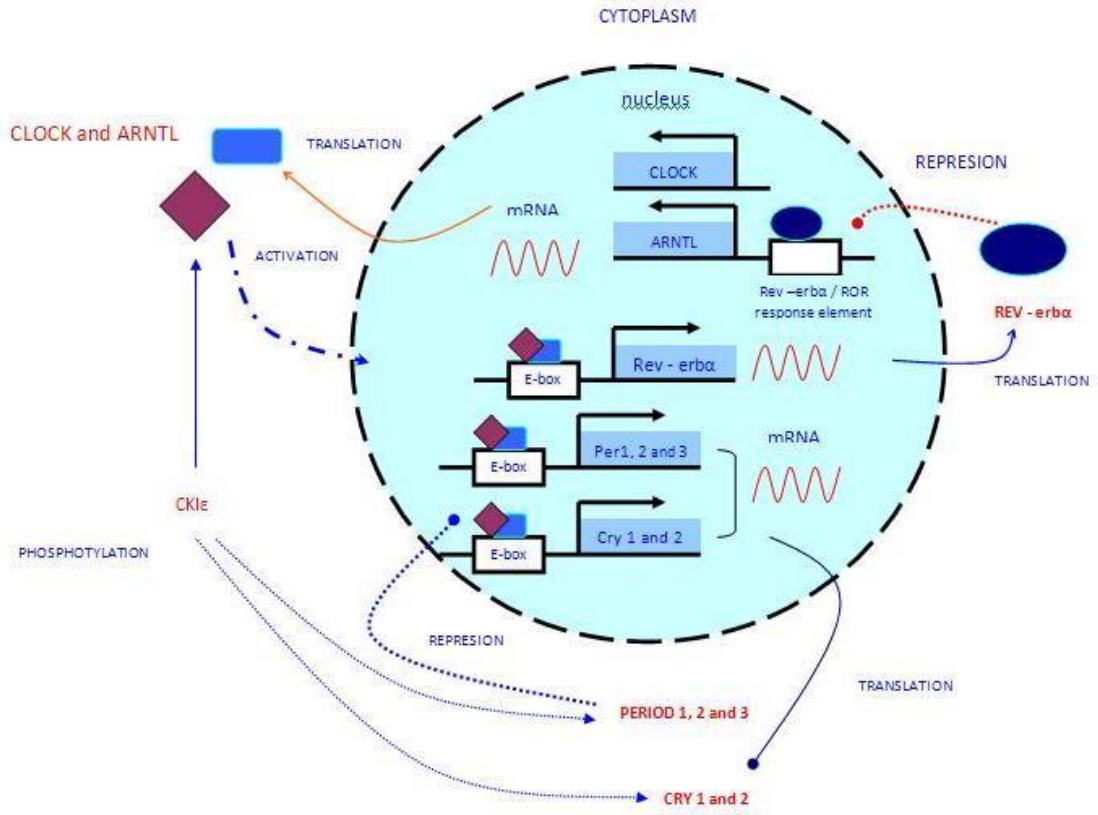

Figure 2. The circadian molecular clock in mammals (Beccket and Roden, 2009: 416)
Positive loop is afforded by CLOCK/ARNTL heterodimer complex which re-enter the nucleus and initiate transcription of the period genes (Per 1,2 and 3) and cryprochrome genes (Cry 1 and 2) through an E-box. Proteins from these genes complex with casein kinase, re-enter the nucleus and inhibit CLOCK/ARNTL induction. CLOCK/ARNTL also inititate the expression of Rev-erbα and Rora in opposite phases and their proteins inhibit ARNTL transcription. These transcriptional-translational loops take ~24 h to complete a cycle and constitute a circadian molecular clock.
Slika 2: Molekularna cirkadiana ura pri sesalcih (Beccket in Roden, 2009: 416)
Pozitivna zanka je regulirana s CLOCK/ARNTL heterodimernim kompleksom, ki ponovno vstopa v jedro in aktivira izražanje periodično aktivnih genov (Per1, 2 in 3) in kriprokromnih genov (Cry1

in 2) preko dejavnika E-box. Beljakovine, ki jih kodirajo ti geni, se vežejo s kaseinsko kinazo in ponovno vstopijo v jedro, kjer zavirajo aktivacijo CLOCK/ARNTL kompleksa. CLOCK/ARNTL kompleks tudi aktivira ekspresijo genov Rev-erbα in Rora genov v nasprotnih fazah, beljakovinski produkti pa inhibirajo prepisovanje gena ARNTL. Ta transkripcijsko-translacijski cikel poteka okrog 24 ur in sestavlja cirkadiano molekularno uro sesalcev.

2.4.3 Transcriptomic studies in finding candidate genes for male infertility, hypothesis free approach

'Omics' approaches, enabling different perspective all contribute in revealing the processes underlying disease and transcriptome studies have already demonstrated their potential (von Kopylow et al., 2010; Johnston et al., 2005; Pilch and Mann, 2006; Deepinder et al., 2007). Transcriptome studies allows simultaneous detection of the expression of almost all genes, thereby enabling complete insight into gene regulation linked to molecular pathogenesis and homeostatic mechanisms, and gene alterations on the transcriptomal level (He et al., 2006).

To date, few studies have used global expression profiling to gain clues to the idiopathic male infertility. In the study of Feig et al. (2007) focus was on the identification of genes that are involved in different stages of germ cell development. Their analyses resulted in a set of 1181 genes which had differential testicular expression through the various stages of germ cell development. They concentrated on three groups that characterized the pre-meiotic, post-meiotic and terminal differentiation stage, which differentiated in biological processes, pathways and transcription factor binding sites (TFBS). In the pre-meiotic stage the highest changes in expression were determined for NF-Y gene, as well as insulin-degrading enzyme and ras related nuclear protein (RAN) binding protein 9. The post-meiotic stage was characterized with over-representation of HLF and Pax6 binding sites, while terminal differentiation was characterized with preeminence of FREAC4 and c-fos binding sites. Differential expression of germ cell specific genes was also demonstrated in the study of Ellis et al. (2007) and loss of germ cell transcript correlated with the increase of inflammatory related genes. In order to identify the specific pathophysiology and molecular pathways of the disease in NOA patients, microarray analyses were also performed on biopsied testes showing a wide range of heterogeneity (Okada et al., 2008). Unlike in the study of Feig et al. (2007) samples were classified on the basis of Johnsen's score, they subgrouped samples according to an unsupervised classification algorithm, the non negative matrix factorization (NMF) method. With the NMF method, NOA samples were separated into three subclasses and 149 transcripts were identified as differentially expressed genes among them. They identified ART3 gene as a susceptibility gene for NOA.

That a considerable contribution of genes in somatic testicular cells is required for normal spermatogenesis was indicated in the study of Spiess et al. (2007). The levels of a minimum of 188 transcripts increased with the severity of spermatogenic failure and reached maximum levels in samples with Sertoli cell-only appearance. The study demonstrated an increase of differentially expressed inflammation-related genes. They showed that mast cells that have been already implicated in many diseases may also play a role in human testicular pathophysiology.

The use of various approaches of grouping testicular pathologies in the previous studies showed inconsistent results, and therefore, information of the groups of genes that proved to be reliable and that could be used as potential biomarkers for male infertility is still not sufficiently understood. Previous studies mainly focused on detection of differential gene expression in germ cells, while possible alterations of gene expression in somatic cells has not yet been sufficiently investigated. Except in the study of Spiess et al. (2007), where the role of mast cells in pathophysiology of infertility has been indicated, possible transcriptomic changes and their effects on spermatogenesis in the Sertoli and Leydig cells are still unknown. Although the substantial role of those cells during all phases of spermatogenesis is indisputable, global gene expression in the Sertoli and Leydig cells was not investigated so far.

2.4.3.1 Human spermatogenesis

Human spermatogenesis is a long and complex process, controlled by hormonal interplay, differential gene expression and cell-cell communication (Wistuba et al., 2007). During spermatogenesis, processes that transform basic spermatogonia into highly specialized mature spermatozoa involve mitosis, meiosis and extensive morphological differentiation. Several developmental stages in forming mature spermatozoa start with undifferentiated cells of germ line type A spermatogonia and spermatogonial stem cells. Except from ensuring self-regeneration, spermatogonial stem cells provide type B spermatogonia. After differentiation and mitotic division, spermatogonia type B evolve into primary spermatocytes that undergo meiosis and produce haploid secondary spermatocytes. After one more genomic reducing step secondary spermatocytes divide into haploid spermatids. In this stage, spermatids drastically change their shape and content. DNA condensation, decrease of cytoplasm, acrosome formation and transformation of shape lead to achieving the typical form of spermatozoa. Although spermatogenesis represents the maturation of germ cells, it is dependent on other cell types. Indispensable endocrine and genomic regulation are supported and mediated by somatic cell types, the Sertoli cells in the tubules and the peritubular myoid cells, as well as Leydig cells in the testicular interstitium.

2.4.3.2 Role of Sertoli cells in spermatogenesis

Sertoli cells are highly specialized cells of the testis, fundamental for their formation and for the normal functioning of spermatogenesis. From the involvement in testis development (Wilhelm et al., 2007) Sertoli cells associate with germ cells and are necessary for their progression into spermatozoa (Griswold, 1998). Located in the seminiferous tubules, these cells have multiple functions:

- represent supporting cells and form a blood - testis barrier which keeps spermatozoa from getting into the blood circulation or the lymphatic system, thus preventing the immune system to create an autoimmune response against the sperm (Dym et al. 1970)
- produce seminiferous tubule fluid which enables the transport of testicular products to the epididymis, nutrition of germ cells and of spermatozoa, and transport of signals required for germ cell-germ cell and germ cell-Sertoli cell communication

- regulate the transport of molecules and proteins and directly synthesizing molecules required for nutrition, proliferation and maturation of germ cells (Jégou, 1993)
- are involved in the migration and apoptosis of germ cells and
- partaking in endocrine regulation of spermatogenesis; the action of FSH and testosterone occurs through Sertoli cells (Walker and Cheng, 2005).

It is known that irregularities in Sertoli cells are associated with male infertility, and that some genes are involved in their normal function. SOX8 is an important regulator of the Sertoli cell function and it is fundamental for the maintenance of adult male fertility (O'Bryan et al., 2008). SOX8 knock-out mice manifested progressive degeneration of seminiferous epithelium, showing that SOX8 is an essential regulator for interactions with developing germ cells. Similar, the absence of SOX9 leads to late male sterility phenotype (Barrionuevo and Scherer, 2010). Loss of the androgen receptor (AR) in the Sertoli cells mainly affects Sertoli cell functions to support and nurture germ cells, resulting in the spermatogenesis arrest at the diplotene primary spermatocyte stage prior to the accomplishment of the first meiotic division (Wang et al., 2009). The loss of function of Katanal1 results in male-specific infertility through disruption of microtubule dynamics and premature exfoliation of spermatids from the seminiferous epithelium (Smith et al., 2012). The study of Sun et al. (2013) showed that metastasis-associated protein1 (MTA1) is essential for the proper contact between Sertoli cells and neighboring germ cells. Patients with impaired spermatogenesis had lack of expression of MTA1 in the Sertoli cells, suggesting the role of this molecule in the control of reproductive homeostasis. The deletion of CX43 in Sertoli cells causes multiple modifications in the testicular gene expression in prepubertal mice primarily influencing germ cells (Giese et al., 2012).

2.4.3.3 Role of Leydig cells in spermatogenesis

Leydig cells, located in the testicular interstitium, produce testosterone and other androgens necessary for spermatogenesis and the development of the male genital tract (Ewing et al., 1981). Testosterone and other androgens are produced in response to luteinizing hormone which binds to the receptor on the surface of Leydig cells. Whereas only Sertoli cells in the seminiferous tubules have receptors for testosterone, these cells are the major targets of hormonal signals that regulate spermatogenesis (Walker and Chang, 2005). Leydig cells also secrete many non-steroidal factors required for the stimulation of seminiferous tubule contraction, beta endorphin for inhibition of Sertoli cell proliferation and function as well as EGF, which is important for the regulation of spermatogenesis (Bostwick and Cheng, 2008).

Loss of the AR in Leydig cells has a major influence on Leydig cells steroidogenic functions resulting in spermatogenesis arrest predominately at the round spermatid stage (Wang et al., 2009). The study of Li et al. (2012) revealed that NDRG2 gene plays an important role in the process of Leydig cell apoptosis, suggesting a possible role in male fertility (Li et al., 2012). Gonzalez et al. (2010) indicated that Leydig cell hyperplasia is probably the result of the increased expression of TGF-beta 1 system (Gonzalez et al., 2010). Inducible nitric oxide (NO) inhibits Leydig cell testosterone production in the rat testis, suggesting a potential role in the inflammation-mediated infertility (O'Bryan et al.,

2000). COUP-TFII is essential for Leydig cell formation and COUP-TFII deletion in the prepubertal mice has been shown to cause infertility, hypogonadism and spermatogenic arrest (Qin et al., 2008). Gonadotropin-regulated testicular RNA helicase e(GRTH/DDX25), except in germ cells present in the Leydig cells, is essential for the completion of spermatogenesis (Tsai-Morris et al., 2010).

2.4.4 Environmental factors contribution in male infertility

Environmental factors play an important role in the increase in infertility risk (Oliva et al., 2001; Sharpe, 2000). It is known that the male reproductive function is highly vulnerable to many chemical and physical agents induced by industrial or agricultural activities. Exposure to certain pesticides, metals and solvents have been linked to the impairment of male fertility (Bonde and Giwercman, 1995). Also changes in many aspects of lifestyle influence the reproductive function. Lifestyle factors like tobacco, alcohol, caffeine, illegal drugs and stress may deteriorate male fertility by impeding spermatogenesis, sperm motility, sperm DNA, chromatin integrity, hormonal regulation or by reducing the fertilizing ability of spermatozoa (Kumar et al., 2009).

3 MATERIAL AND METHODS

3.1 ASSOCIATION ANALYSIS OF GENETIC VARIATION IN CIRCADIAN RHYTHM GENES CLOCK AND ARNTL WITH MALE INFERTILITY

3.1.1 Ethics statement

The study was approved by committees in both countries participating in the study: Slovenian National medical ethics committee (reference number: 73/05/12) and by the Ethics committee at Medical faculty at University of Belgrade, Serbia (reference number: 29/II-3). All patients gave informed written consent to participate in the study.

3.1.2 Subjects

Male partners of infertile couples, attending the infertility outpatient clinic of the Department of Obstetrics and Gynecology, University Medical Centre Ljubljana, and the Institute of Human Genetics, and Institute of Urology and Nephrology, Faculty of Medicine, University of Belgrade, participated in the study. The diagnosis of infertility was based on clinical assessment (testicular volume was measured using Prader's orchidometer), semen analysis, follicle stimulating hormone (FSH) testing and histologic evaluation of testicular biopsy specimens. Exclusion criteria were: patients with a history of testicular carcinoma, obstructive azoospermia including congenital bilateral absence of vas deferens, cytogenetic abnormalities and Y chromosome microdeletions.

The control group of proven fertility consisted of 444 healthy males, who were fathers of at least one child and who reported no history of infertility. The seminal parameters and biopsy were not assessed for subjects in the control group. All patients and control subjects were Caucasian of the Slavic (Slovene or Serbian) origin and were recruited in a consecutive manner.

3.1.2.1 Semen parameters

In the 517 infertile men, semen analysis was performed according to the World Health organization (WHO) criteria (Laboratory ..., 1999). Sperm was assessed in terms of volume, concentration, rapid progressive motility and normal morphology.

3.1.2.2 Hormonal parameters

Serum FSH was measured by Microparticle Enzyme Immunoassay (AxSYM System, Abbott Laboratories, Chicago, IL, USA); the reference interval for FSH was 1–8 mIU/ml.

3.1.2.3 Testicular biopsy and histological evaluation

Testicular biopsy and histological evaluation was performed in 77 patients with diagnosis of non obstructive azoospermia. In all patients testicular biopsy was performed for diagnostic and therapeutic (sperm recovery followed by cryostorage) purposes.

Following unilateral hemiscrototomy, a small incision was made and at least two samples of testicular tissue were taken from each testis. A systematic histological evaluation was

performed under light microscopy; more than 100 seminiferous tubules were scored for each patient and results expressed as a relative number of tubules showing Sertoli cells, spermatogonia, spermatocytes, round and elongated spermatids, and spermatozoa. The diagnoses were as follows: Sertoli-cell only syndrome (SCOS) alone 46.8 %, Maturation arrest (MA) alone 11.3 %, Germinal hypoplasia (GH) alone 7.8 %, and following combinations: GH+MA 14.1 %, SCOS+MA 11.3 %, GH+SCOS+MA 6.3 % and GH+SCOS 2.1 %.

3.1.3 SNP selection and genotyping

Genetic variants in both key circadian rhythm-regulating genes, CLOCK and ARNTL, were genotyped in this study. Eight tagging single nucleotide polymorphisms (SNPs) were chosen from both genes. Of these, four SNPs, rs11932595, rs6811520, rs6850524, and rs13124436 were selected in the CLOCK gene, and four SNPs, rs3789327, rs1481892, rs4757144, and rs12363415 in the ARNTL gene.

During the study design, we aimed to estimate the contribution of genetic variation in CLOCK and ARNTL genes in the most comprehensive way possible. We have taken gene size into consideration and were aware of the large sizes of both CLOCK (119 kb) and ARNTL (110 kb) genes. However, during SNP selection process, we have also utilized valuable information on genetic linkage landscape originating from HapMap and 1000genomes projects. Information on genetic linkage in the two selected genes allowed us to genotype most informative (tagSNPs) SNPs in this region, but also to omit genotyping redundant proxy SNPs with large extent of linkage association to already selected tagSNPs.

To accomplish this, data on genetic linkage in regions of CLOCK and ARNTL along with 10kb flanking regions were acquired from HapMap dataset (population CEU, release 29, from February 2009) and analyzed using the Tagger algorithm available through the Haploview software (Haploview, version 4.2) (de Bakker et al., 2005). We observed considerable rates of genetic linkage in the region of the ARNTL gene (Figure 3), with one block of linkage spanning close to 50 % of the gene region, and two larger blocks spanning most of the other 50 % of ARNTL size. Tagger results revealed that using a set of 4 SNPs we could capture variation for 59 SNPs residing in ARNTL with power (r^2) exceeding 80 %. To maximize statistical power, only the SNPs with minor allele frequency values exceeding 0.05 were included in the investigated set.

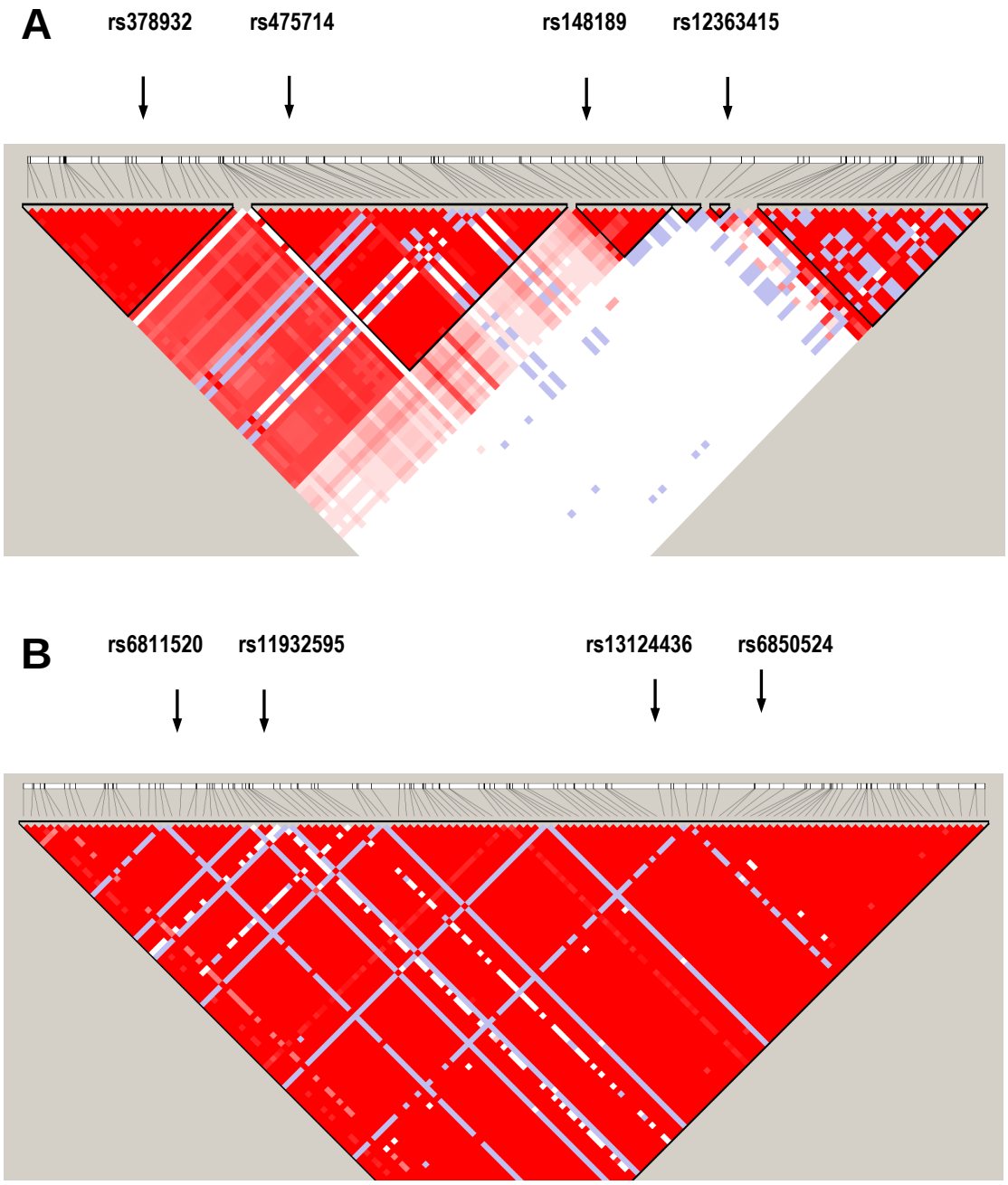

Figure 3: Patterns of linkage disequilibrium in ARNTL and CLOCK genes
(A) Profile of linkage disequilibrium in the ARNTL gene. A large block of linkage, covering almost 50 % of the ARNTL gene region, followed by a stretch of 2 large LD blocks and two smaller ones in between.
(B) Large extent of genetic linkage was observed in the CLOCK gene, which was located in one large block of linkage disequilibrium. The colors represent D' pairwise linkage (red represents high linkage, white represents absence of linkage).
Slika 3: Vzorec genetske vezave v genih ARNTL in CLOCK

(A) Profil genetske vezave v genu ARNTL. Razviden je obsežen blok vezanih polimorfizmov, ki pokriva skoraj 50 % genomske regije ARNTL gena in mu sledita dva večja bloka vezanih polimorfizmov.
(B) Velik obseg genetske vezave je prisoten tudi v regiji gena CLOCK, ki se v celoti nahaja v enem obsežnem bloku genetske vezave. Barve na grafih predstavljajo obseg genetske vezave (rdeča barva pomeni izrazito genetsko vezavo, bela predstavlja odsotnost vezave).

3.1.3.1 DNA isolation and SNPs genotyping

Genomic DNA was isolated from the peripheral blood samples using standard procedures. SNPs genotyping was carried out by real time PCR method performed on 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using KASPar SNP genotyping chemistry. The PCR reaction mix of 8 µL final volume consisted of:

- 4 µL of DNA sample
- 4 µL of Reaction Mix 2X
- 0.11 µL Assay Mix
- 0.064 µL 50mM MgCl2
- 3.826 µL H2O.

The protocol for PCR amplification is presented in Table 2.

Table 2: Protocol for PCR amplification
Preglednica 2: Postopek pomnoževanja s PCR

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	94 °C	15 minutes	1x
Denaturation, annealing and extension	94 °C 57 °C or 61 °C 72 °C 94 °C 57 °C or 61 °C	10 seconds 5 seconds 10 seconds 10 seconds 20 seconds	20x
Final extension	72 °C	40 seconds	1x

The allelic discrimination analysis was performed using SDS Software Version 1.2 (Applied Biosystems, Foster City, CA, USA).

Genotype assignment was performed and interpreted independently by two investigators.

3.1.4 Estimation of differential expression for ARNTL and CLOCK genes

Considering that we have performed global transcriptome analysis in the ensuing steps of this study, we also estimated the expression of the two genes in data from microarray profiling. We have extracted the expression ratios (M-values) for the two genes and estimated the expression differences based on data originating from microarray probes A_23_P162037 and A_23_P419038 that interrogated expression of ARNTL and CLOCK genes, respectively.

3.1.5 Statistical analysis

Significances of associations between allelic and genotype frequencies and disease status were analyzed using the Chi-square test ($\chi 2$). Odds ratios (OR) and their respective 95 % confidence intervals (CI) were also calculated to compare allelic and genotype distribution in patients and control subjects. Analyses were performed using R statistical language (R 2.15.0 for Windows). $\chi 2$ goodness-of-fit tests for deviation of genotype distribution from those predicted by Hardy-Weinberg equilibrium were also calculated, providing an additional quality control step of genotyping process. The investigated associations were regarded as significant when they reached $p \leq 0.05$.

As multiple SNPs were investigated, appropriate corrections of significance values were also applied using the Benjamini-Hochberg correction method (false-discovery rate – FDR values). We studied the extent of genetic linkage between SNPs using Haploview software version 4.2, which enables calculation of D' and r2, representing the extent of pairwise linkage between two SNPs.

Haplotype frequencies and haplotype-disease associations were estimated using haplo.stats package for R (Schaid et al., 2002). Functions in haplo.stats package permit the calculation of indirectly measured haplotypes, under the founding assumption that subjects in the study are not related and the linkage phase unknown (Schaid et al., 2002). Here, the *haplo.score* function was used to directly ascertain differences in haplotype distributions across the groups of infertile men and control individuals. A global test of association, and per-haplotype association test were performed for both investigated genes. To reduce the effects of multiple testing we excluded all haplotypes with a frequency below 5 % from downstream tests.

For power calculations, the *pbsize2* function belonging to the *gap* package for R was utilized, which allows accurate power estimations under a variety of disease inheritance models (Zhao et al., 2007).

3.2 TRANSCRIPTOME ANALYSIS OF HUMAN TESTIS GENE EXPRESSION

3.2.1 Ethics statement

The study was approved by Slovenian National medical ethics committee (reference number: 73/05/12). All patients gave informed written consent to participate in the study.

3.2.2 Experimental design

The study included 20 samples (10 showing obstructive azoospermia and 10 samples showing non obstructive azoospermia). Differential gene expression was measured in relation to the Agilent's Universal Human Reference RNA and each experimental sample was hybridized against this common reference sample.

3.2.3 Patients

All biopsy samples were obtained from patients attending the outpatient infertility clinic of the Andrology Centre, Department of Obstetrics and Gynecology in Ljubljana. The study population was composed of 20 infertile patients with azoospermia who attempted testicular biopsy either for obstructive azoospermia or non obstructive azoospermia.

Before sperm recovery, medical history was established, testicular volume measured and serum FSH level was assessed.

3.2.3.1 Testicular biopsy

Testicular biopsy was performed under local anaesthesia. Following unilateral hemiscrototomy, a small testicular incision was made and at least two samples of testicular tissue were taken from each testis. The first sample was fixed in Bouin's solution, routinely embedded in paraffin and cut at a section thickness of 5 μm. The sections were stained with HE, PAS and van Gieson–Weigert. A systematic histological evaluation was performed under light microscopy. More than 100 seminiferous tubules were scored for each patient. The results were expressed as a relative number of tubules showing Sertoli cells, spermatogonia, spermatocytes, round and elongated spermatids, and spermatozoa. All examinations were made by the same observer (J.S.). The diagnoses were as follows: normal spermatogenesis, hypospermatogenesis, early and late maturation arrest and Sertoli-cell-only sindrome (SCOS).

In all patients testicular biopsy was performed for diagnostic and therapeutic (sperm recovery followed by cryostorage) purpose. Additionally, in the six patients epididimovasostomy was attempted. Ductal system patency was obtained in all 6 patients with normozoospermia in 3 to 6 months after surgery.

3.2.3.2 Histological and morphometrical analysis, volume density of Sertoli cells and Leydig cells

The step serial sections were histologically analysed and morphometrically evaluated. For histological analysis, hematoxylin – eosin staining method was used. Volume density of Sertoli cells and Leydig cells was morphometrically evaluated. Morphometrical analysis was performed on a Wild sampling microscope (Wild, Heerbrugg, Switzerland), using Weibel's B 100 double grid test system (Weibel ER., 1979). Volume density of interstitial tissue (Sertoli cells or Leydig cells) was estimated by counting points of grid system, which hit interstitial tissue (Sertoli cells or Leydig cells) and reference space at an objective magnification of 40x (reference space — hits on interstitial tissue and myocytes).

Volume density of interstitial tissue (VVi) is the quotient between hits falling on interstitial tissue (Pi) and hits falling on reference space (Pt) (VVi = Pi/Pt). Volume density of interstitial tissue is expressed in mm^3/mm^3. The histopathologist, who was not aware of the clinical status and group of the subject, evaluated the slides.

3.2.3.3 RNA isolation

Total RNA was isolated from frozen testis biopsy samples by using the TissueLyserLT (Qiagen) apparatus and RNeasy Plus Micro kit (Qiagen) according to the manufacturer's instructions. About 3.5-4.5 mg weight of tissue was disrupted and homogenized in 2 ml centrifuge tubes containing 5-mm stainless steel beads and lysis buffer (Buffer RLT plus) at a frequency of 50 Hz for 10 minutes in a TissueLyser device. Homogenate was subsequently centrifuged for 3 minutes, collected and eluted in a gDNA eliminator spin column. Ethanol was added to the flow-through to provide appropriate binding conditions for RNA and samples were then applied to an RNeasy MinElute spin columns, where total RNA was bound to the membrane and contaminants were efficiently whased away. RNA was then eluted in 14µl of water. RNA concentrations were determined using NanoDrop 2000C Spectrophotometer (Thermo Scientific) and RNA quality was verified by Agilent Bioanalyzer 2100 (Agilent Technologies).

3.2.4 Microarray experiment and data analyses

Measure of expression was performed on isolated RNA from biopsy samples of patients with obstructive and non obstructive azoospermia using Agilent Whole Human Genome 4x44 microarrays (Agilent design id: 14850). Gene expression platform contains 264x10 biological probes, targeting altogether 19.596 unique mRNA sequences according to NCBI Reference Human Genome Build version 33. A single kit contains four glass slides, each formatted with four high-definition 44k arrays. For labeling reaction, 50 ng of RNA from samples was used. Sample preparing, labeling and amplification RNA (Low Input Quick Amp Labeling Kit, two color, Agilent Technologies), hybridization, washing and scanning were performed according to the manufacturer's recommendations (Agilent Technologies). Hybridization was performed for 17 h, rotating at a speed of 10 rpm at 65 °C in an hybridization oven (Agilent technologies).

After hybridization, microarray slides were scanned using Agilent High Resolution Microarray Scanner System, using the manufacturer's recommended scanning settings. Subsequently, microarray features were extracted using Agilent Feature Extraction software v10.7.3.1. Background was substracted using Agilent's background detrend algorithms, features with non uniform fluorescence profile were removed from further analyses and linear lowess intra-array approach was used to account for and correct possible dye bias. Consistency of log ratio values was inspected using manufacturers provided spike-in probes and reproducibility was evaluated by investigating the extent of variance in the population of replicated probes. Further analysis steps were performed using limma package from the Bioconductor software v2.8 package in R statistical environment version 2.13.1. Processed flouorescence values obtained in preceding steps were read-in using slightly modified limma function. The complete dataset was then inspected for NA values and other irregularities. Probe annotations were obtained from the

Agilent's eArray service (earray.chem.agilent.com) and appended to initial datasets. Before calculating log ratio values between flourescence signals originating from each color channel, all the values were offset by 100 units, to prevent the undesirable increase in variance in the population of features with low flouorescence values, ultimately causing abnormalities in MA diagnostic plots. Statistical comparisons of expression values were evaluated using moderated t-test approach implemented in limma. This test accounts for the issue of small variance contributing to undesirably large t-test values occuring for some genes, and uses Bayes inference to acknowledge similarities in gene expression profiles in subsets of genes, thereby improving prioritization of genes with differences best related to frank biological alterations. A linear model was set up, incorporating the effect of disease status and gender, and expression values were fit to this model. Significance values and log fold-change were calculated afterwards and p-values were controlled for the issue of multiple testing by the method related to that described by Benjamini and Hochberg (Benjamini et al., 1995) and implemented into the limma workflow.

3.2.5 csSAM analyses

In order to maximize the information obtainable from heterogeneous testis tissue cell type-specific significance analysis of microarrays (csSAM) was performed (Shen-Orr et al., 2010). Given data from heterogeneous tissue regarding to information about the relative abundance of each cell type of each sample, the csSAM methodology statistically deconvolves cell-type specific expression profiles and uses them to adjust for heterogeneity, performing cell-type-specific differential expression analysis.

3.2.6 Quantitative RT-PCR analyses

The expression levels of sixteen selected differentially expressed genes were validated by quantitative real-time PCR (qRT-PCR) on fifteen independent samples, seven with severely impaired spermatogenesis and eight with normal spermatogenesis. Testicular RNA was used as a template in first-strand cDNA synthesis with SuperScript® VILO™ cDNA Synthesis Kit.

The protocol for cDNA synthesis was as follows:
5X VILO™ Reaction Mix 4 µl
10X SuperScript® Enzyme Mix 2 µl
RNA (up to 2.5 µg) x µl
DEPC-treated water to 20 µl
Incubation at 25 °C for 10 minutes.
Incubation at 42 °C for 60 minutes.
Termination the reaction at 85 °C at 5 minutes.

Primer pairs for each gene were designed by PrimerBank. The sequences of the primer pairs used for each gene are shown in the Table 3. Primer optimization and efficiencies were performed prior to the relative quantitation of the expression of the genes.

Table 3: The sequences of the primer pairs used in the study
Preglednica 3: Zaporedja začetnih oligonukleotidov uporabljenih za merjenje ekspresije genov z metodo PCR v realnem času

Gene	Forward primer	Tm (°C)	Reverse primer	Tm (°C)
SPATA18	GCAGGAAAAGCTAGACTTCTGG	60.6	TGGCAACTTGCTCAATGAGTTC	61.3
FSCB	ATGGTAGGCAAATCCCAGCAA	61.8	GGGTAGCTTTGGGGCTAGATG	62.0
TEKT1	GGCACATTGCTAACAAGAACCA	61.3	CTCTGGCTTTCTGCGACCA	62.0
PRKAA1	TTGAAACCTGAAAATGTCCTGCT	60.6	GGTGAGCCACAACTTGTTCTT	60.7
SMAD2	CGTCCATCTTGCCATTCACG	61.4	CTCAAGCTCATCTAATCGTCCTG	60.2
CREB1	ATTCACAGGAGTCAGTGGATAGT	60.2	CACCGTTACAGTGGTGATGG	60.4
FTMT	TGGAGTGTGCTCTACTCTTGG	60.9	ACGTGGTCACCTAGTTCTTTGA	61.0
RNF168	GGATCTGCATGGAAATCCTCG	60.6	ACTGGAAGCACGGTTTACACA	61.9

Real-time qRT–PCR (Applied Biosystems, Foster City, CA, USA) was performed using the SYBR® Green RT PCR Reagents Kit (Applied Biosystems) in triplicate in a 11 µl PCR reaction. cDNA (1 µl) was used in a 11 µl PCR mixture containing 5.5 µl of SYBR® Green (Applied Biosystems), 4.4 µl of NF H2O and 0.1 µl of 1 mM primer for each gene.

The protocol for qRT-PCR amplification is presented in Table 4.

Table 4: Protocol for qRT-PCR amplification
Preglednica 4: Postopek pomnoževanja qRT-PCR

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	95 °C	10:00	1x
Denaturation, annealing and extension	95 °C 57 °C 72 °C	0:15 0:30 0:30	50x
Final extension	95 °C 60 °C 95 °C	0:15 0:20 0:15	1x

The relative expression of each gene was determined on the basis of the C_T value. The relative expression level of the genes of interest was computed relative to the endogenous controls, GAPDH and ACTINB, to normalize for variances in the quality of RNA and the amount of input cDNA.

3.2.7 Meta-analysis of transcriptomic alterations in testicular biopsy tissue samples from infertile males

To maximise the potential of our study for discovery of consistent expression alterations in male infertility, we included data from a variety of microarray studies that interrogated global gene expression alterations in testicular biopsy tissue samples from infertile males. The main inclusion criteria for studies were availability of raw datasets at either GEO or ArrayExpress repositories, common reference design approach towards measuring differential expression in the microarray experiment and interrogation of the same tissue samples and comparable disease states as in our experiment. Two previously performed microarray studies have met these criteria and we therefore included resulting raw data on gene expression alterations on testicular biopsy tissue samples from infertile males and performed a meta-analysis of these datasets.

All the steps described in following sections were performed in using R statistical language version 2.9.2, using Bioconductor environment. Raw data from microarray experiments was obtained from Gene Expression Omnibus (GEO) repository (Sean and Meltzer, 2007) and from ArrayExpress repository. Selection of datasets considered for inclusion, along with further details is presented in the table below (Table 5).

Table 5: Description of datasets included in the meta-analysis
Preglednica 5: Izbor in opis študij za vključitev v meta-analizo podatkov

Dataset accession	Number of samples - azoospermia	Number of samples – obstructive forms of infertility	Array platform	Number of probes on the array
GSE9210	47	11	Agilent-012097 Human 1A Microarray (V2) G4110B	~22000 probes
GSE4797	5	18	GE Healthcare/Amersham Biosciences CodeLink™ UniSet Human 20K I Bioarray	~23000 probes
E-MEXP-1019*	17	13	In-house printed array using the Illumina RefSet human oligonucleotide collection (ArrayExpress design accession: A-MEXP-693)	~16000 probes

* This dataset was ultimately excluded due to using paired sample design used in hybridization of microarrays instead of common reference design used in our and other studies included in the meta-analysis.
* Označeni podatkovni set je bil iz nadalnjih analiz izključen zaradi uporabe pristopa parnega testiranja vzorcev, za razliko od uporabe pristopa skupnega referenčnega vzorca, ki smo ga uporabili v naši študiji in drugih dveh študijah vključenih v meta-analizo.

Two GEO datasets with accession numbers GSE9210 and GSE4797 in adddition to our own microarray results were finally included meta-analysis. Raw datasets were put through quality control steps implemented in arrayQualityMetrics package, followed by intra-array quantile and inter-array loess normalization, where necessary. Afterwards intensities of probe replicates targeting specific transcripts were median averaged and only values for transcripts interrogated in all three studies were retained through further meta-analysis steps.

Differential expression of genes across all three studies was calculated using meta-analysis algorithms implemented in the RankProd package (Hong et al., 2006). RankProd uses a non-parametric approach that selects genes displaying consistently highly ranked across different microarray studies and is therefore a feasible meta-analytic tool across a variety of microarray studies originating from different laboratories and performed under differing conditions (Hong et al., 2006). We utilized *RPadvance* function, with *origin* parameter set to account for data originating from three different sources. P values were calculated using permutation approach (1000 permutations) and predicted false positive rates estimated across permutation cycles (Reiner et al., 2003).

4 RESULTS

4.1 ASSOCIATION ANALYSIS OF GENETIC VARIATION IN CIRCADIAN RHYTHM GENES CLOCK AND ARNTL WITH MALE INFERTILITY

4.1.1 Clinical, sperm and hormonal characteristics of the infertile patients

Clinical, sperm and hormonal characteristics of the 517 infertile patients are summarized in Table 6.

Table 6: Clinical, sperm and hormonal characteristics of the 517 infertile men
Values are given as means ± SD.
Preglednica 6: Klinične lastnosti, semenski in hormonski parametri pri 517 neplodnih moških vključenih v našo študijo
Vrednosti so navedene kot povprečja ± SD.

Clinical parameters	Non obstructive azoospermia (n=219)	Oligoasthenoteratozoospermia (n=298)			
		0.1-0.9×10^6 sperm/ml (n=112)	≥1-4×10^6 sperm/ml (n=45)	5-19×10^6 sperm/ml (n=71)	≥20×10^6 sperm/ml (n=34)
Testicular volume (right/left) (ml)	10.5 ± 6.5 / 10.4 ± 6.0	12.2 ± 4.8 / 11.9 ± 4.9	12.5 ± 4.5 /13.7 ± 4.8	14.6 ± 4.2 / 14.7 ± 4.9	19.6 ± 4.8 / 19.3 ± 4.7
Sperm concentration (×10^6 sperm/ ml)	0	0.37 ± 0.2	2.9 ± 1.0	8.1 ± 3.4	59.8 ± 46.9
Rapid progressive motility (%)	0	4.2 ± 1.7	10.1 ± 8.1	12.6 ± 10.0	26.3 ± 12.9
Normal morphology (%)	0	5.1 ± 7.1	8.2 ± 8.9	10.3 ± 9.2	24.9 ± 14.4
Serum FSH level (IU/l)	20.9 ± 16.6 (n=201)	11.8 ± 8.9 (n=83)	9.2 ± 5.0 (n=36)	4.1 ± 1.0 (n=47)	2.2 ± 0.9 (n=2)

4.1.2 Effects of analyzed polymorphisms in the CLOCK and ARNTL genes on the risk of male infertility

Genotype frequencies of investigated polymorphisms were in accordance with those predicted by the Hardy-Weinberg equilibrium in the study and in the control group ($p<0.05$), with the exception of rs12363415, which was excluded from further analyses. Genotype and allelic distribution of the CLOCK and ARNTL polymorphisms of the 517 infertile man and 444 fertile controls are shown in Table 7.

Table 7: Genotype and allelic distribution of the CLOCK and ARNTL polymorphisms of the 517 infertile men and 444 fertile controls
Preglednica 7: Razporeditev frekvenc genotipov in alelov genov ARNTL in CLOCK pri populaciji 517 neplodnih moških in 444 plodnih moških

Gene	SNP	Alleles		Infertile male			Controls			P value
		1	2	11	12	22	11	12	22	
CLOCK	rs11932595	G	A	129 (26.16)	235 (47.66)	129 (26.16)	64 (15.45)	201 (48.55)	149 (35.99)	$6 \cdot 10^{-5}$
CLOCK	rs6811520	T	C	66 (14.76)	198 (44.29)	183 (40.93)	59 (15.60)	208 (55.02)	111 (29.36)	$2 \cdot 10^{-3}$
CLOCK	rs6850524	G	C	182 (35.82)	245 (48.22)	81 (15.94)	109 (27.87)	195 (49.87)	87 (22.25)	0.01
CLOCK	rs13124436	G	A	225 (46.10)	216 (44.26)	49 (9.63)	217 (51.05)	170 (40)	38 (8.94)	0.299
ARNTL	rs3789327	T	C	148 (29.6)	237 (47.4)	115 (23)	143 (33.96)	202 (47.98)	76 (18.05)	0.129
ARNTL	rs1481892	G	C	228 (46.43)	221 (45.01)	42 (8.55)	193 (45.62)	200 (47.28)	30 (7.09)	0.637
ARNTL	rs4757144	G	A	107 (21.79)	244 (49.69)	140 (28.51)	111 (26.24)	176 (41.60)	136 (32.15)	0.047
ARNTL*	rs12363415	G	A	26 (6.31)	112 (27.18)	274 (66.50)	29 (6.90)	115 (27.38)	276 (65.71)	0.935

We found a statistically significant difference in the allelic distribution of rs11932595 ($p=6 \cdot 10^{-5}$), rs6811520 ($p=2 \cdot 10^{-3}$) and rs6850524 (p=0.01) (Hodžić et. al., 2013). However, we did not, find any significant association between rs13124436 polymorphism from CLOCK gene and male infertility. Under recessive genotype model, OR estimates ranged between 1.4 and 1.9 for CLOCK gene polymorphisms: rs11932595 ($p=6 \cdot 10^{-5}$, OR=1.9 with 95 % CI 1.4-2.7), rs6811520 ($p=2 \cdot 10^{-3}$, OR=1.7 with 95 % CI 1.2-2.2) and rs6850524 (p=0.01, OR=1.4 with 95 % CI 1.1-1.9).

4.1.3 Effects of inferred haplotypes in the CLOCK and ARNTL genes on the risk of male infertility

We also analyzed the inferred haplotypes in both investigated genes. The frequencies of predicted haplotypes in the study and the control group are presented in Table 8.

Table 8: Frequencies and distribution of probable haplotypes in the patient and control groups as predicted by haplo.stats.
Preglednica 8: Pogostosti in razporeditev določenih haplotipov v populaciji neplodnih in plodnih moških, glede na predikcije algortma haplo.stats.

	CLOCK gene							
	rs6811520	rs6850524	rs11932595	rs13124436	Frequency (patients)	Frequency (controls)	p	Simulated p value
1	T	C	A	G	0.21	0.28	$8 \cdot 10^{-5}$	$5 \cdot 10^{-5}$
2	C	G	A	G	0.05	0.06	0.49	0.48
3	C	G	G	A	0.09	0.09	0.20	0.20
4	C	G	A	A	0.13	0.10	0.14	0.14
5	C	G	G	G	0.20	0.17	0.04	0.04
6	C	C	G	G	0.07	0.04	$8 \cdot 10^{-3}$	$8 \cdot 10^{-3}$
	ARNTL gene							
	rs3789327	rs1481892	rs4757144	rs12363415	Frequency (patients)	Frequency (controls)	p	Simulated p value
1	T	G	G	A	0.11	0.12	0.28	0.28
2	T	G	A	A	0.21	0.22	0.62	0.62
3	T	C	G	A	0.10	0.11	0.73	0.72
4	C	G	G	A	0.08	0.08	0.69	0.70
5	C	G	A	A	0.15	0.12	0.07	0.07
6	C	C	G	A	0.06	0.04	0.07	0.06

A statistically significant difference in haplotype distributions was also confirmed at the CLOCK gene locus when comparing the frequencies of haplotypes TCAG ($p=8 \cdot 10^{-5}$, simulated p value after 10.000 permutations was $5 \cdot 10^{-5}$), CCGG ($p=8 \cdot 10^{-3}$, simulated p value was $8 \cdot 10^{-3}$) and CGGG (p=0.04, simulated p value equaled to 0.04) between the infertile patients and fertile controls.

The SNPs interrogated in the ARNTL gene, rs3789327, rs1481892 and rs4757144 did not show significant associations of genotype or allelic distribution between the two groups.

Accordingly, we did not find any significant difference comparing the frequencies of 6 most frequent haplotypes for the 4 analyzed SNPs in the ARNTL gene in the study and control groups.

Power analyses were performed to estimate the lower sensitivity threshold of our study to detect the variants characterized by low-to-modest effect sizes. For this purpose, the pbsize2 function of gap package for R was utilized. Calculations showed that our power to detect a significant result in the presence of the actual genotype-phenotype effect with genotype relative risk equal to at least 1.7, was 82.3 %, when taking into account the sample size, the significance threshold of 0.05, the prevalence of male infertility in the general population equal to 4.5 %, and disease allele frequency of at least 10 %, and considering multiplicative model of genetic association.

4.1.4 Estimation of differential expression for ARNTL and CLOCK genes

Comparison of expression levels for ARNTL and CLOCK gene based on the microarray profiling results described in the following sections, has also shown significant diffences in expression of these two genes in testicular biopsy samples from patients with severe spermatogenesis disturbances in comparison to controls with normal spermatogenesis.

We have detected a significant down-regulation of CLOCK gene expression in patients with impaired spermatogenesis (logFC value: -0.80, P value: 0.002) and a concomitant up-regulation of ARNTL gene expression (logFC value: 0.63, P value: 0.0002) compared to controls with normal spermatogenesis (Figure 4).

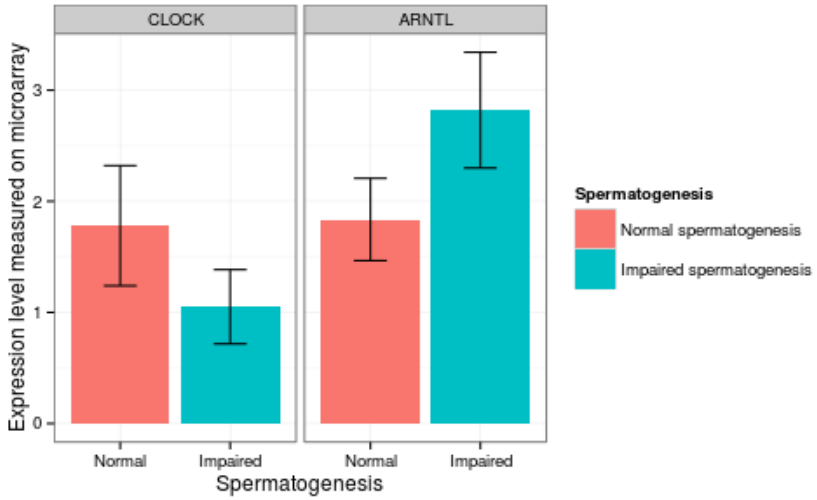

Figure 4: Expression levels of CLOCK and ARNTL gene in individuals with impaired spermatogenesis in comparison to those with normal spermatogenesis
Slika 4: Nivoji izražanja genov ARNTL in CLOCK pri posameznikih z moteno spermatogenezo v primerjavi s posamezniki z normalno spermatogenezo.

4.2 TRANSCRIPTOME ANALYSIS OF HUMAN TESTIS GENE EXPRESSION

4.2.1 Patients characteristics

Patients characteristics are presented in Table 9.

Table 9: Clinical characteristics of patients included in the transcriptome profiling study
Preglednica 9: Klinične lastnosti neplodnih moških v transkriptomsko študijo

Patient	Clinical findings*	Testicular volume (ml)	FSH level (IU/l)	Sperm count[a]	Sperm motility[b]	Histology**	Johnsen's score
1	bilateral epididymal enlargement	12/13	3,7	3	2	normal spermatogenesis	10
2	bilateral epididymal enlargement	25/28	4,75	3	2	normal spermatogenesis	9
3	bilateral epididymal enlargement	13/16	3,2	3	2	normal spermatogenesis	9
4	bilateral epididymal enlargement	22/25	7,02	3	2	normal spermatogenesis	9
5	CABVD	14/14	3,63	3	2	normal spermatogenesis	9
6	CABVD	28/28	1,68	2	0	normal spermatogenesis	9
7	enlarged right epididymis	15/15	8,3	3	2	Hypospermatogenesis, mononuclear infiltration	9
8	enlarged right epididymis 8 years after	15/15	2,2	3	2	hypospermatogenesis	9
9	no enlargement	25/25	1,79	2	1	hypospermatogenesis	9
10	enlarged right epididymis	30/30	1,27	2	2	hypospermatogenesis, EMA	9
11	NA	NA	NA	0	0	LMA	6
12	NA	15/15	12,5	0	0	SCOS, EMA, hypospermatogenesis	5
13	NA	20/20	14,2	0	0	SCOS, EMA	5
14	NA	12/15	12,8	0	0	SCOS, EMA	5
15	NA	8/10	13	0	0	SCOS, EMA	5
16	hypogonadism	1/1	82	0	0	SCOS, EMA	2
17	hypogonadism	6/4	59,8	0	0	SCOS, LCH	2
18	NA	15/15	44	0	0	SCOS, EMA	2
19	NA	8/10	28,7	0	0	SCOS, EMA	1
20	hypogonadism	2/2	36,4	0	0	SCOS, LCH	1

*CABVD, congenital bilateral absence of vasa deferentia.
**EMA, early maturation arrest. LMA, late maturation arrest. LCH, Leydig cell hyperplasia. NA, not available.
[a]0 = no sperm; 1 = rare; 2 = low; 3 = numerous. [b]0 = immotile; 1 = non-progressively motile; 2 = progressively motile
*CABVD, kongenitalna bilateralna odsotnost semenovodov
**EMA, zgodnji zastoj rasti. LMA, pozni zastoj rasti. LCH, hiperplazija Leydigovih celic. NA, ni na voljo.
[a]0 = ni spermijev; 1 = redki; 2 = nizki; 3 = številni. [b]0 = negiblijivi; 1 = ne-progresivno gibljivi; 2 = progresivno gibljvi.

4.2.2 Histological and morphometrical analysis, volume density of Sertoli cells and Leydig cells

Histological analysis of testis biopsy sample is shown in Figure 5.

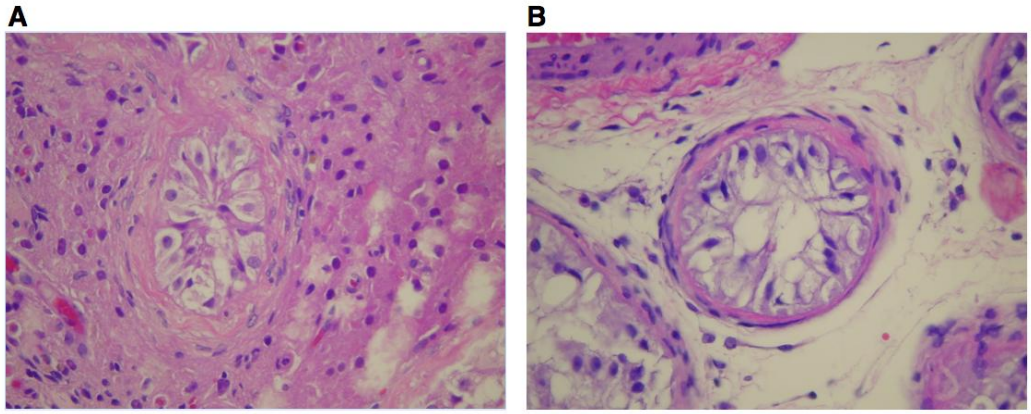

Figure 5: Histological analysis of testis biopsy sample
A. 400x magnification, hematoxilin-eosin. Centrally, the seminiferous tubule is shown with Sertoli cells without any spermatogonia. In the edematous interstitial tissue there are few Leydig cells; B. 400x magnification, hematoxilin-eosin. Centrally, the hialinisation of seminiferous tubule is shown with few Sertoli cells and few spermatogonia. In the interstitial tissue there are several hyperplastic Leydig cells.
Slika 5: Histološka analiza bioptičnega vzorca testisa
A. 400x povečava, barvanje s hematoksilin-eozinom. V osrednjem delu je vidna semenska cevka s Sertolijevimi celicami brez spermatogonijev. V edematozno spremenjenem intesticijskem tkivu je vidnih malo Leydigovih celic.; B. 400x povečava, hematoksilin-eozin. V osrednjem delu je vidna hialinizacija semenske cevke z malo Serolijevimi celicami in maloštevičnimi spermatogoniji. V intersticiju so številne hiperplastične Leydigove celice.

Results of morphometrical analysis, volume density of Sertoli and Leydig cells are shown in Table 10.

Table 10: Volume density of Sertoli cells and Leydig cells
Preglednica 10: Prostorninska gostota Sertolijevih in Leydigovih celic

Patient	Volume density of Sertoli cells (mm^3/mm^3)	Volume density of Leydig cells (mm^3/mm^3)
1	4.72	3.89
2	4.63	3.70
3	3.61	3.47
4	4.72	3.89
5	2.92	4.03
6	4.72	5.00
7	4.44	1.25
8	3.47	2.64
9	not available	not available
10	3.89	3.75
11	not available	not avaliable
12	8.89	8.06
13	4.72	3.05
14	5.14	6.39
15	6.67	0.56
16	6.11	15.4
17	4.17	10.5
18	7.78	0.69
19	5.83	1.11
20	0.74	7.04

4.2.3 Microarray data pre-processing

Quantile normalization was used to account for inter-array variation in global distribution of fluorescence values, as this method was shown to perform well in comparison to other methods. Probe replicates were median averaged before further steps. Before and after normalization, MA plots, boxplots and expression value density plots were investigated for diagnostic purposes (Figures 5 and 6).

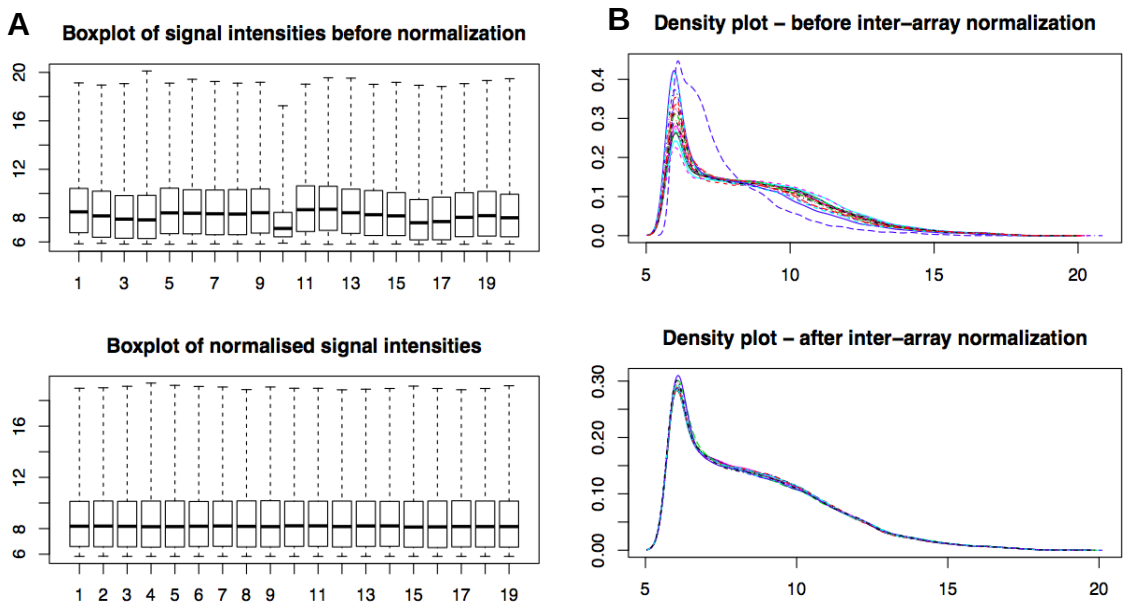

Figure 6: Distribution of microarray signal intensities before and after quantile inter-array normalization in the form of box-plots (A) and density plots (B)
Slika 6: Razporeditev intenzitet signalov na mikromrežah pred in po postopku normalizacije
Grafi so prikazani v obliki škatlastega grafa (A) in gostotnega grafa (B).

Note that one of the arrays was a clear technical outlier (array 10) and was therefore excluded prior to the normalization.

We have also performed microarray quality diagnostics by inspecting MA distribution plots. MA plots represent any potential bias of the array hybridization, washing, scanning or normalization procedure and enable visual inspection of the relatedness of absolute fluorescence values to ratios between red and green signal. Here, no deviations from expected distribution was observed for any of the scanned arrays (Figure 7).

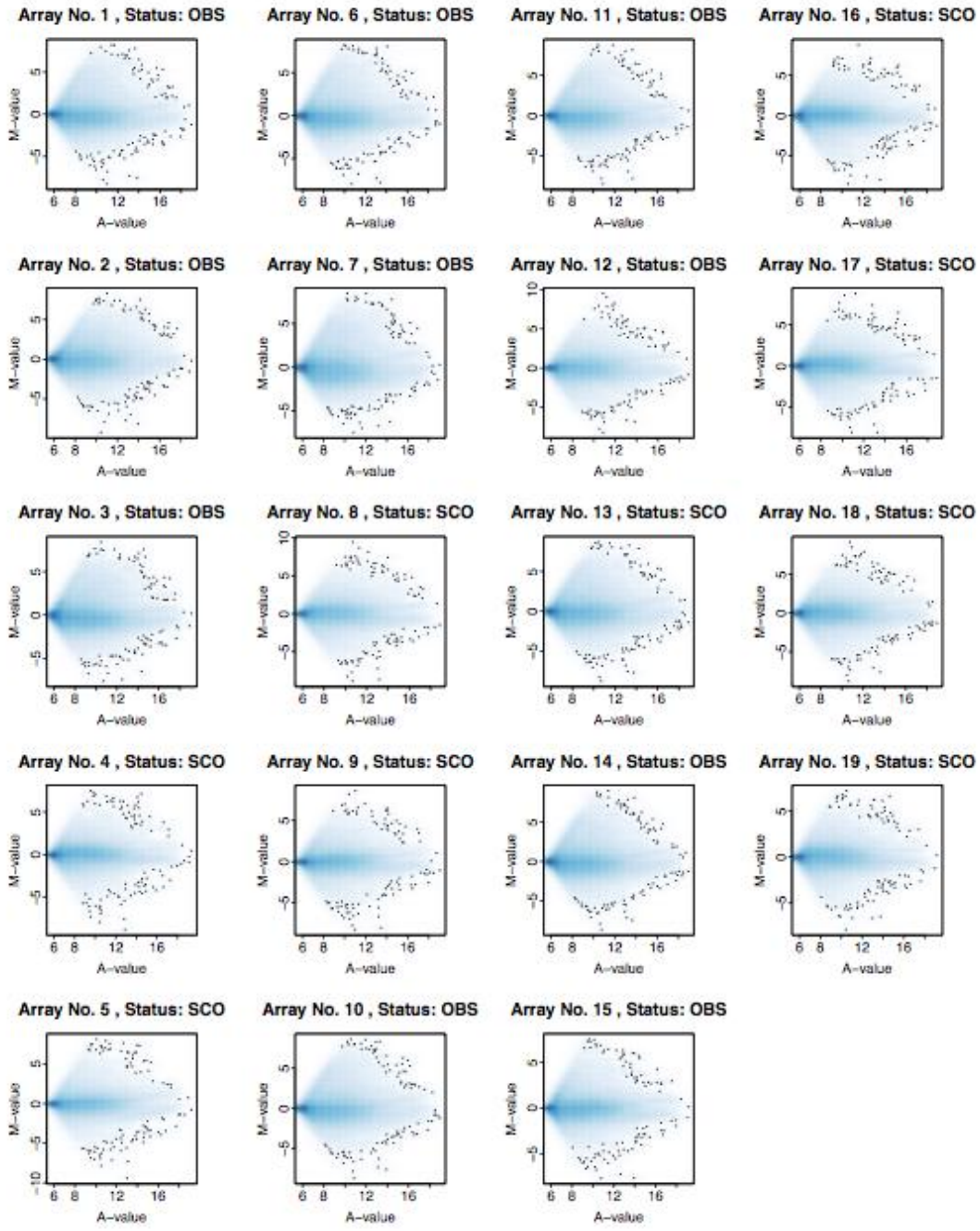

Figure 7: MA diagnostic plots for each of the arrays hybridized and scanned in our global transcription profiling experiment
Slika 7: Grafi MA za posamezne vzorce preiskovane z mikromrežami v poskusih merjenja globalnega izražanja genov pri moški neplodnosti

4.2.4 Analysis results

4.2.4.1 Principle sources of variation in the dataset

To observe what are the greatest sources of variation in all analyzed samples, we performed multidimensional scaling (MDS) on all the arrays investigated. This shows which samples are more similar to each other than to others and allows inspection of potential biases and sources of variation in the experiment (Figure 8).

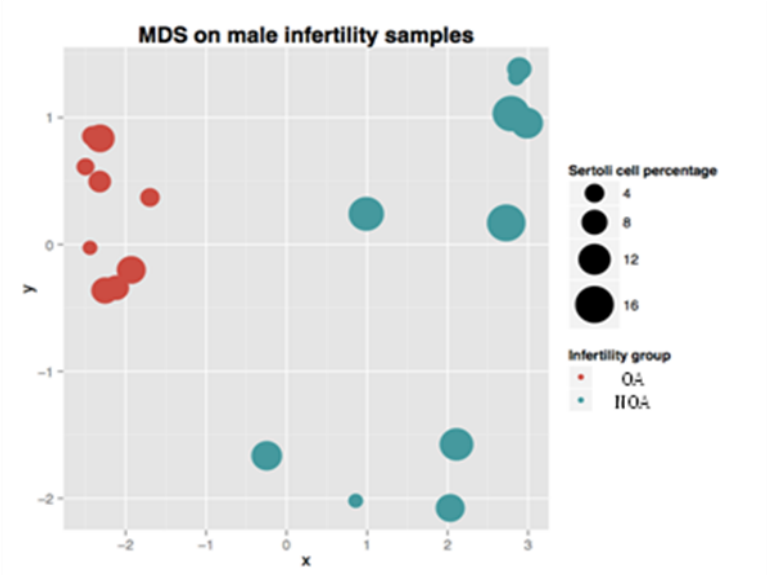

Figure 8: Multidimensional scaling analysis of all arrays examined in the study with presentation of Sertoli cell proportions
Size of dots represents Sertoli cell proportion, colour represents affection status.
Slika 8: Rezultati analize mikromrež z redukcije dimenzij podatkov z mikromrež (MDS) s predstavitvijo deleža Sertolijevih celic
Velikost točke predstavlja delež Sertolijevih celic v bioptičnem vzorcu, barva predstavlja tip moške neplodnosti.

The separation between samples with severely impaired and samples with normal spermatogenesis is clear. There is, however no clear distribution related to the proportion of Sertoli cells in tissue samples.

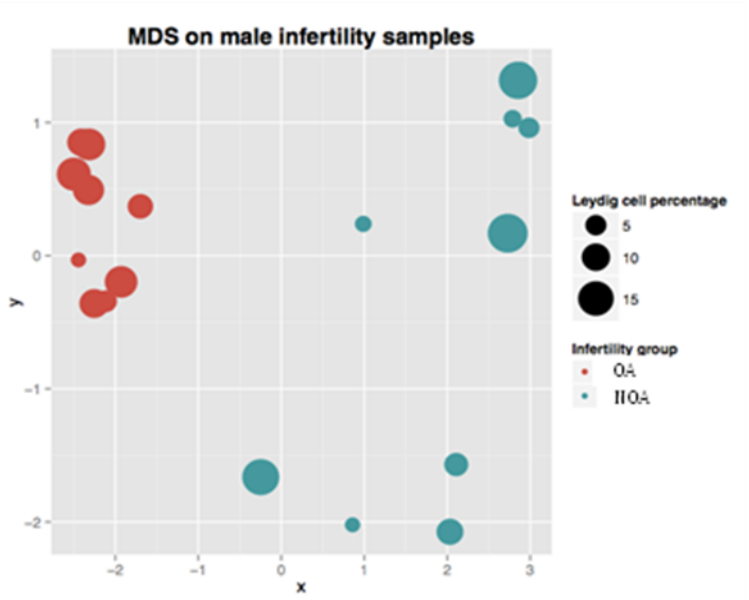

Figure 9: Multidimensional scaling analysis of all arrays examined in the study with presentation of Leydig cell proportions
Size of dots represents Leydig cell proportion, colour represents affection status.
Slika 9: Rezultati analize mikromrež z redukcije dimenzij podatkov z mikromrež (MDS) s predstavitvijo deleža Leydigovih celic
Velikost točke predstavlja delež Leydigovih celic v bioptičnem vzorcu, barva predstavlja tip moške neplodnosti.

The separation between samples with severely impaired and samples with normal spermatogenesis is clear. There is, however no clear distribution related to the proportion of Leydig cells in tissue samples.

4.2.4.2 Microarray analysis of global gene expression changes in patients with severely impaired spermatogenesis

Using Agilent Whole Human Genome 4x44 microarrays (Agilent design id: 14850), we analyzed global gene expression changes in testis biopsy samples from 10 subjects with severely impaired spermatogenesis and 10 subjects with normal spermatogenesis. Excluding one sample identified as outlier left 19 samples for analysis. One of the goals of our study was to identify impaired spermatogenesis associated transcriptomic signatures, and therefore we investigated the extent to which the groups of sample with severely impaired and normal spermatogenesis differ from one another with respect to the overall gene expression patterns. With a strict statistical criterion (adj. $p < 0.00001$) we identified more than 6000 differentially expressed genes. Analysis of expression levels of the significantly changed genes showed that the majority of the genes were down-regulated in subjects with severely impaired spermatogenesis, compared with subjects with normal spermatogenesis. Of these 6000, 4362 were down-regulated and 1638 were up-regulated. We identified 471 genes with at least 4-fold down-regulated expression in the subjects with severely impaired spermatogenesis compared to subjects with normal spermatogenesis.

4.2.4.3 Gene set enrichment analyses

To gain more insight into key processes that may possibly explain functional differences among the testicular samples from severely impaired and normal spermatogenesis types, we carried out functional annotation analysis of the top differentially expressed genes, attaining adjusted p-values values below 0.01 and using hypergeometric test, based on genes' relation to GeneOntology and KEGG terms. The analysis revealed an enrichment of Gene Ontology (GO) terms related to mitotic and meiotic cell cycle, spermatid differentiation and development, microtubule organization and germ cell development (Table 11).

Table 11: GeneOntology top enriched terms
Preglednica 11: Seznam najvišje uvrščenih bioloških procesov genske ontologije GeneOntology

GOBPID	Term	Pvalue
GO:0000226	microtubule cytoskeleton organization	3.9E-10
GO:0000086	G2/M transition of mitotic cell cycle	2.3E-07
GO:0007059	chromosome segregation	1.4E-06
GO:0007286	spermatid development	2.0E-06
GO:0031023	microtubule organizing center organization	4.0E-06
GO:0000209	protein polyubiquitination	1.1E-05
GO:0043161	proteasomal ubiquitin-dependent protein catabolic process	1.4E-05
GO:0007018	microtubule-based movement	3.1E-05
GO:0051297	centrosome organization	3.8E-05
GO:0034453	microtubule anchoring	3.8E-05
GO:0000075	cell cycle checkpoint	4.2E-05
GO:0000236	mitotic prometaphase	4.7E-05
GO:0007128	meiotic prophase I	5.4E-05
GO:0071156	regulation of cell cycle arrest	7.5E-05
GO:0007127	meiosis I	8.4E-05
GO:0030317	sperm motility	1.1E-04
GO:0051324	prophase	1.5E-04
GO:0009566	fertilization	1.5E-04
GO:0006323	DNA packaging	1.5E-04
GO:0007281	germ cell development	1.6E-04
GO:0071103	DNA conformation change	1.8E-04
GO:0046051	UTP metabolic process	2.0E-04
GO:0051321	meiotic cell cycle	2.0E-04
GO:0007338	single fertilization	2.2E-04
GO:0007126	meiosis	2.4E-04
GO:0051327	M phase of meiotic cell cycle	2.4E-04

KEGG pathway analysis showed an enrichment of functional categories including protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis, lysosome, phagosome and Escherichia coli infection which correspond to inflammation and autoimmune activity (Table 12).

Table 12: KEGG top enriched terms
Preglednica 12: Seznam najvišje uvrščenih terminov iz baze KEGG

KEGGID	Term	P value
4141	Protein processing in endoplasmic reticulum	1.67E-06
4120	Ubiquitin mediated proteolysis	3.59E-06
4110	Cell cycle	3.35E-05
4114	Oocyte meiosis	1.22E-04
4142	Lysosome	1.38E-04
5130	Pathogenic Escherichia coli infection	1.60E-03
4145	Phagosome	3.33E-03
4520	Adherens junction	6.02E-03
4530	Tight junction	6.88E-03
4914	Progesterone-mediated oocyte maturation	8.52E-03

4.2.4.4 IPA pathway analyses

To further characterize the functional consequences of gene expression changes associated with impaired spermatogenesis, we performed pathway analysis of the gene expression data with Ingenuity pathway analysis (IPA). Several networks were identified as significantly associated with the differentially expressed genes. Among the most prominent networks, noticeable are networks related to cell cycle, cell signaling, cellular organization, urological development and function as well as DNA replication, recombination and repair. The three top ranked networks are shown in the Figures 10, 11 and 12.

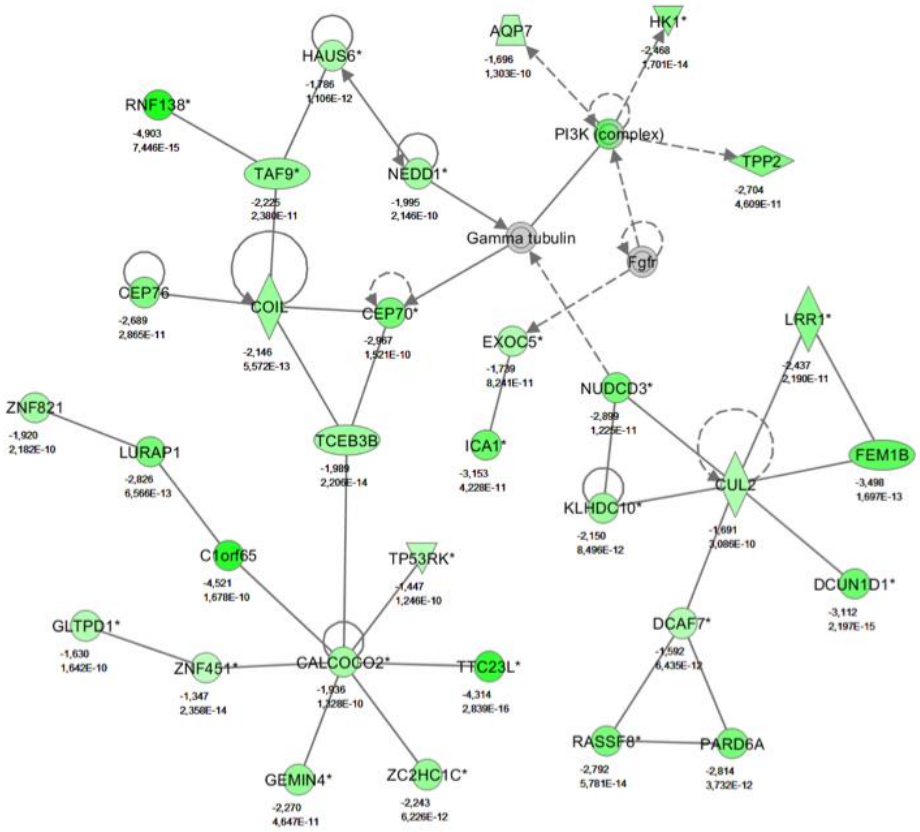

Figure 10: Network 1, related to Cell cycle, Cell signaling, Cellular assembly and organization

Genes are represented by either green or red circles corresponding to their down-regulation and up-regulation, respectively. Color intensity of circles is correlated to the size of expression differences (logFC values). Numbers below the gene labels represent logFC and significance values for differential expression.

Slika 10: Omrežje 1, povezano s celičnim ciklom, celično signalizacijo in organizacijo celične strukture

Geni so na shemi prikazani v obliki krožcev, kjer zelena barva predstavlja zvišano izražanje, rdeča pa znižano izražanje v testisu moških z motnjo v spermatogenezi. Intenzivnost barve predstavlja velikost spremembe ekspresije (logFC vrednosti). Vrednosti pod imeni genov predstavljajo logFC vrednosti in P vrednosti za razlike v izražanju.

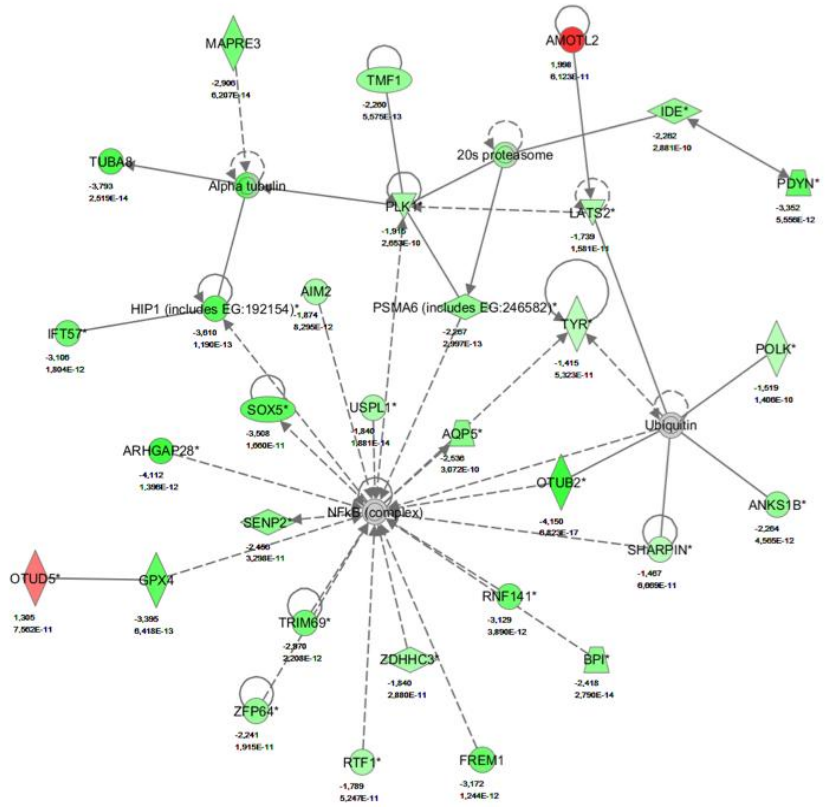

Figure 11: Network 2, related to Cell-to-cell signaling, Urological development and function

Genes are represented by either green or red circles corresponding to theirdown-regulation and up-regulation, respectively. Color intensity of circles is correlated to the size of expression differences (logFC values). Numbers below the gene labels represent logFC and significance values for differential expression.

Slika 11: Omrežje 2, povezano z medcelično signalizacijo in razvojem ter funkcijo urološkega sistema

Geni so na shemi prikazani v obliki krožcev, kjer zelena barva predstavlja zvišano izražanje, rdeča pa znižano izražanje v testisu moških z motnjo v spermatogenezi. Intenzivnost barve predstavlja velikost spremembe ekspresije (logFC vrednosti). Vrednosti pod imeni genov predstavljajo logFC vrednosti in P vrednosti za razlike v izražanju.

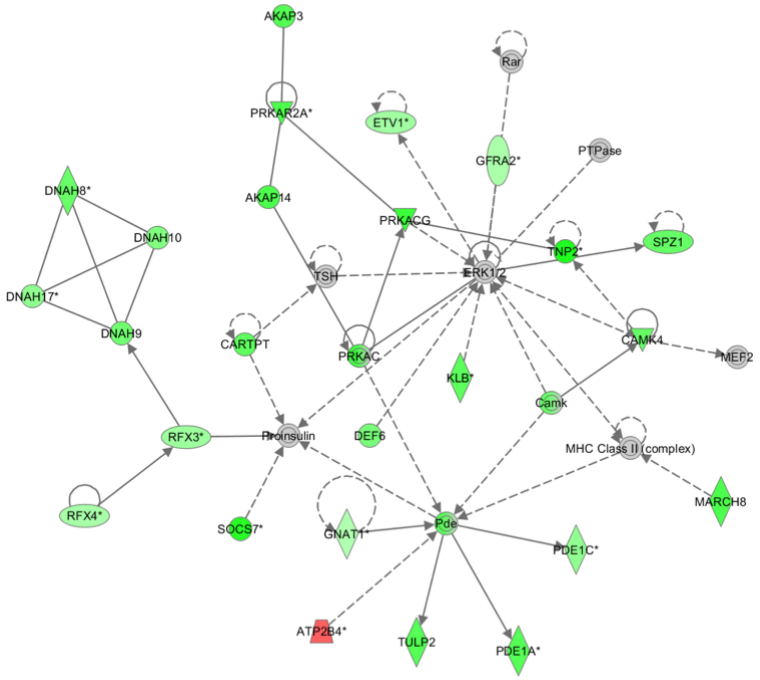

Figure 12: Network 3, related to DNA replication, Recombination and Repair
Genes are represented by either green or red circles corresponding to theirdown-regulation and up-regulation, respectively. Color intensity of circles is correlated to the size of expression differences (logFC values). Numbers below the gene labels represent logFC and significance values for differential expression.
Slika 12: Omrežje 3, povezano z replikacijo DNA, rekombinacijo in popravilom DNA
Geni so na shemi prikazani v obliki krožcev, kjer zelena barva predstavlja zvišano izražanje, rdeča pa znižano izražanje v testisu moških z motnjo v spermatogenezi. Intenzivnost barve predstavlja velikost spremembe ekspresije (logFC vrednosti). Vrednosti pod imeni genov predstavljajo logFC vrednosti in P vrednosti za razlike v izražanju.

To obtain a more detailed picture of genes constituting the three top ranked networks we used Biomedical knowledge discovery server (A.M.L. Liekens et al., 2011). Given the names of networks genes and query: male infertility, we prioritized genes in relation to the given concept. BioGraph revealed that the three top ranked networks consist of genes that almost all interact with Aurora kinase C gene (AURKC). BioGraph also showed that networks contain genes that are associated with CFTR gene.

IPA reveals two signifficant upstream regulatory factors acting on genes differentially expressed. ADCYAP1 and PAPOLB were inhibited, whereas cycloheximide, fulvestrant and FIGLA were activated (Figure 13).

Upstream Regulator	Log Ratio	Predicted Activation State	Activation z-score	p-value of overlap
ADCYAP1 (includes EG:11516)	-0,591	Inhibited	-2,324	1,00E00
PAPOLB	-2,234	Inhibited	-2,000	2,17E-04
MYC	0,582		-1,996	1,00E00
HOXD10	0,768		-1,982	2,84E-01
D-glucose			-1,972	1,00E00
CREM (includes EG:12916)	-4,101		-1,293	2,04E-02
cycloheximide		Activated	2,200	1,00E00
fulvestrant		Activated	2,455	1,00E00
FIGLA		Activated	2,740	7,64E-07

Figure 13: Upstream factors in regulation of differentially expressed genes
Results from IPA Upstream Regulator analysis, which was used to identify the cascade of usptream transcriptional regulators that can explain the observed gene expression changes. In the table regulation statistics is presented for top upstream regulatory factors. The activation z-score is used to infer likely activation states of upstream regulators based on comparison with a random regulation assignment model. P-value of overlap represents the significance of overlap between differentially expressed genes in our dataset with that regulated by the transcriptional regulators.
Slika 13: Zgornji regulatorni dejavniki genov s spremenjenim izražanjemRezultati IPA analize zgornjih dejavnikov, ki je bila uporabljena za analizo kaskade zgornjh dejavnikov, ki v največji meri pojasnijo spremembe ugotovljene v globalnem profilu ekspresije pri naših vzorcih. V tabeli so predstavljeni najvišje uvrščeni dejavniki glede na razultate analize. Aktivacijska vrednost z odseva predvideno stanje zgornjega regulatornega dejavnika, na osnovi primerjave z modelom naključnega pripisovanja stanja aktivacije dejavnikov. P-vrednost prekrivanjapredstavlja statistični pomembnosti preseka med različno izraženimi geni v naši študiji in regulatornimi tarčami zgornjih dejavnikov.

4.2.4.4 Cell-specific SAM analyses

In order to define differentially expressed genes in the Sertoli and Leydig cell populations between the samples with severely impaired and samples with normal spermatogenesis we performed cell-specific analyses. Here, we compared the differential expression of genes, after matrix deconvolution based on cell proportions of Leydig and Sertoli cells (Shen-Orr et al., 2010). This enables the cell specific inspection of differential expression.

We can observe that whole tissue SAM analyses give higher differential expression significances than those performed in sub-cell populations (Figure 14). Observing the whole tissue we can see that more than 10000 genes reaching significant differential expression values. With csSAM analysis for Sertoli cells we identified less than 1000 differentially expressed genes with p value under 0.2, while for Leydig cells we identified less than 1000 differentially expressed genes with p value under 0.4.

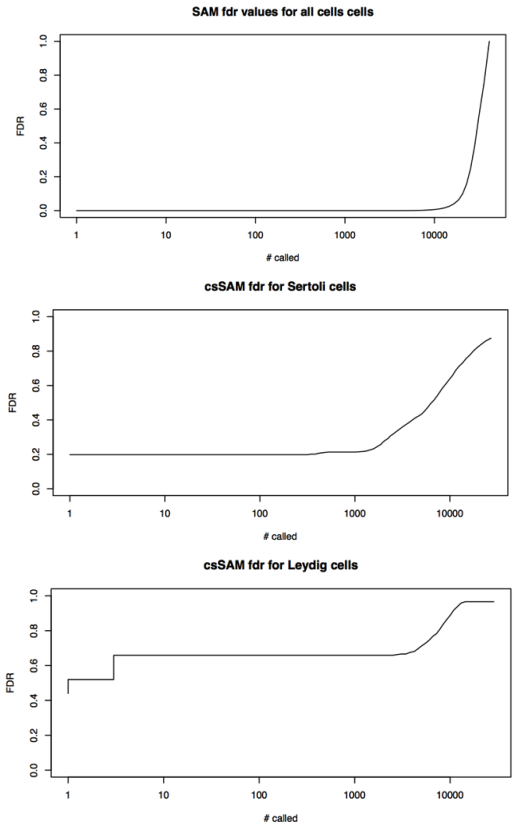

Figure 14: csSAM results plots, x-axis represents the number of genes reaching FDR threshold and y-axis represents the size of FDR threshold
Slika 14: Rezultati analize z algoritmom csSAM, kjer os x predstavlja število genov ki dosegajo prag FDR, os y pa predstavlja višino praga FDR

4.2.4.5 qRT-PCR validation

We selected a set of differentially expressed genes for biological validation on fifteen independent samples. Genes selected for validation were chosen among the top differentially expressed genes from our experiment. Selected groups of genes were functionally related to spermatogenesis, infertility or SOX9 gene.

The qRT-PCR results confirmed trend of down-regulation of all validated genes on the set of samples with severely impaired spermatogenesis (Figure 14). Eight genes showed significant differences ($p<0.05$) between groups with severely impaired and normal spermatogenesis.

Genes FTMT ($p<0.02$), SPATA18 ($p<0.02$), FSCB ($p<0.01$), PRKAA1 ($p<0.01$), RNF168 ($p<0.003$), TEKT1 ($p<0.01$) were chosen according to their p values and highest fold change, while SMAD2 ($p<0.008$) and CREB1 ($p<0.01$) gene were chosen according the functional association with SOX9 by protein interaction.

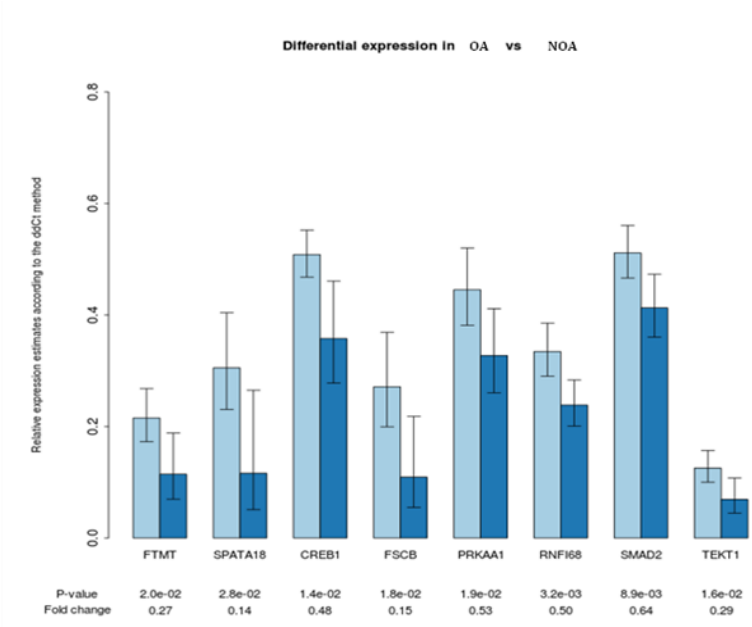

Figure 15: Differential expression of 8 selected genes in patients with severely impaired and normal spermatogenesis
Slika 15: Razlike v izražanju 8 izbranih genov pri bolnikih s hudimi motnjami spermatogeneze v primerjavi z bolniki z normalno spermatogenezo

Cluster analysis of the expression levels of the 8 selected genes showed that the majority of the genes were down-regulated in samples with severely impaired spermatogenesis, compared with samples with normal spermatogenesis (Figure 16).

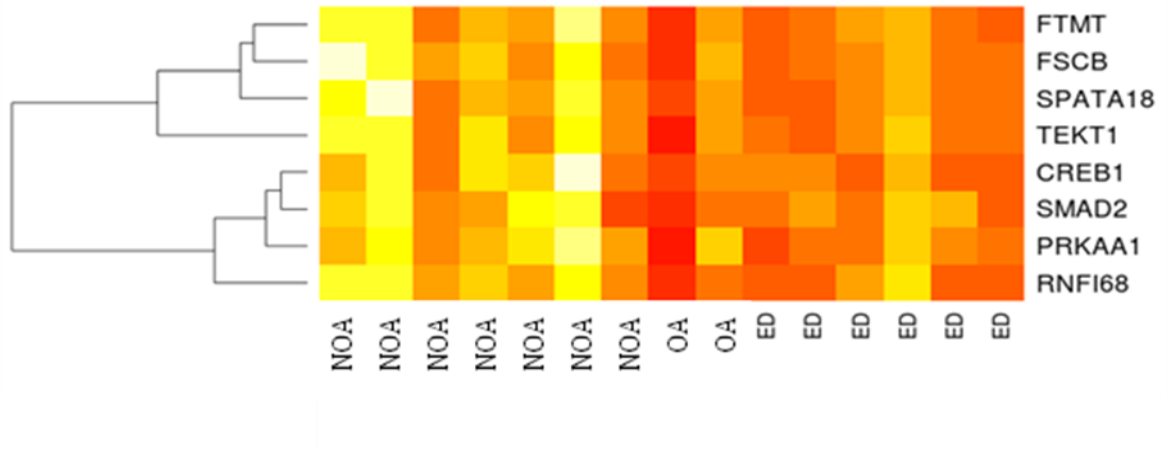

Figure 16: Comparison of gene expression patterns between patients with severely impaired and normal spermatogenesis
The heatmap show the expression profiles of 8 differentially expressed genes between patients with impaired and normal spermatogenesis. Each column represents a sample (NOA, non obststructive

azoospermia; OA, obstructive azoospermia; ED, ejaculation disorders) and each row represents a gene.

Slika 16: Primerjava vzorca izražanja genov med posamezniki s hudimi motnjami v spermatogenezi v primerjavi s pozamezniki z normalno spermatogenezo

Toplotna karta prikazuje vzorce izražanja 8 izbranih genov. Posamezen stolpec predstavlja posamezen vzorec (NOA, neobstruktivna azoospermia; OA, obstruktivna azoospermia; ED, motnje ejakulacije), vrstica predstavlja posamezen gen. Rdeča barva predstavlja višje izražanje gena, rumena nižje izražanje gena.

Next, principal component analysis (PCA) was performed to better visualize the differences in expression of the 8 genes in relation to the stage of disease. Such analysis of the set of independent samples confirmed that the 8 genes were able to separate almost all subjects with severely impaired spermatogenesis from subjects with normal spermatogenesis (Figure 17).

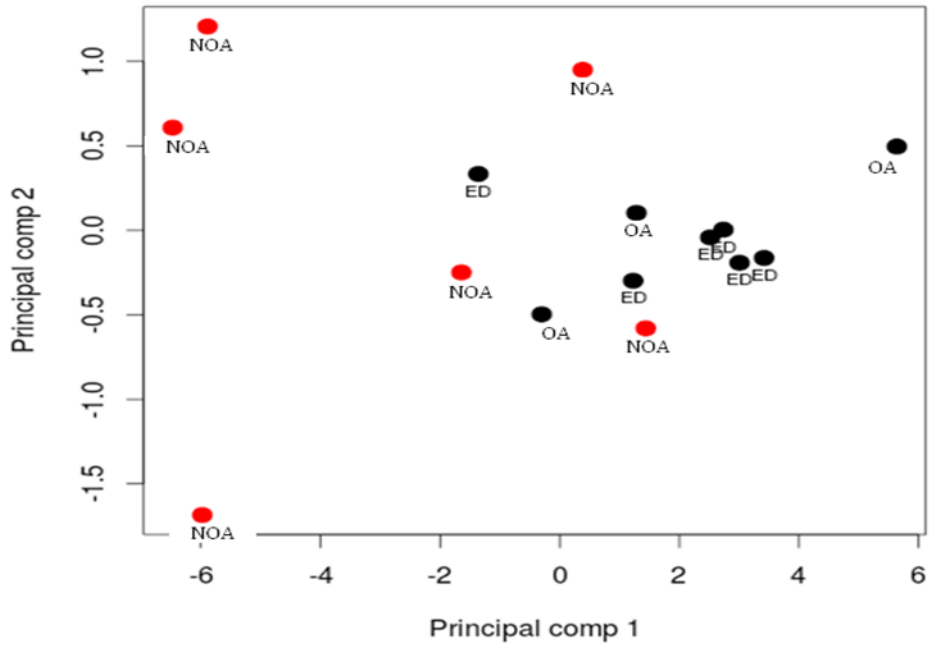

Figure 17: Principal component analysis of differences in expression of the 8 gene in relation to the stage of disease

Red dots represents patients with severely impaired spermatogenesis (NOA, non obstructive azoospermia) and black dots represents patients with normal spermatogenesis (OA, obstructive azoospermia; ED, ejaculation disorders).

Slika 17: Analizi poglavitnih komponent variance na profilih izražanja 8 izbranih genov v povezavi z obliko neplodnosti

Rdeče točke predstavljajo paciente s hudo motnjo primarne spermatogeneze (NOA, neobstruktivna azoospermia), črne predstavljajo paciente z normalno spermatogenezo (OA, obstruktivna azoospermia, ED, motnje ejakulacije).

4.3 META-ANALYSIS OF TRANSCRIPTOMIC ALTERATIONS IN TESTICULAR BIOPSY TISSUE SAMPLES FROM INFERTILE MALES

Altogether, expression levels of 8644 GenBank transcripts were profiled across all three included studies (two GEO previously performed datasets and in our own gene expression profiling study) and could be included in the summary analyses across three studies. Results of meta-analysis have shown that 1933 (22.4 %) transcipts were found significantly up-regulated and 1791 (20.7 %) transcripts down-regulated consistently, attaining PFP values below or equal to 0.05. High concordance between results of the three studies was found, in terms of direction of gene differential expression as well as selection of most significantly differentially expressed genes (Figure 18). The list of top 10 genes attaining highest differential expression across the three studies is presented in Table 13.

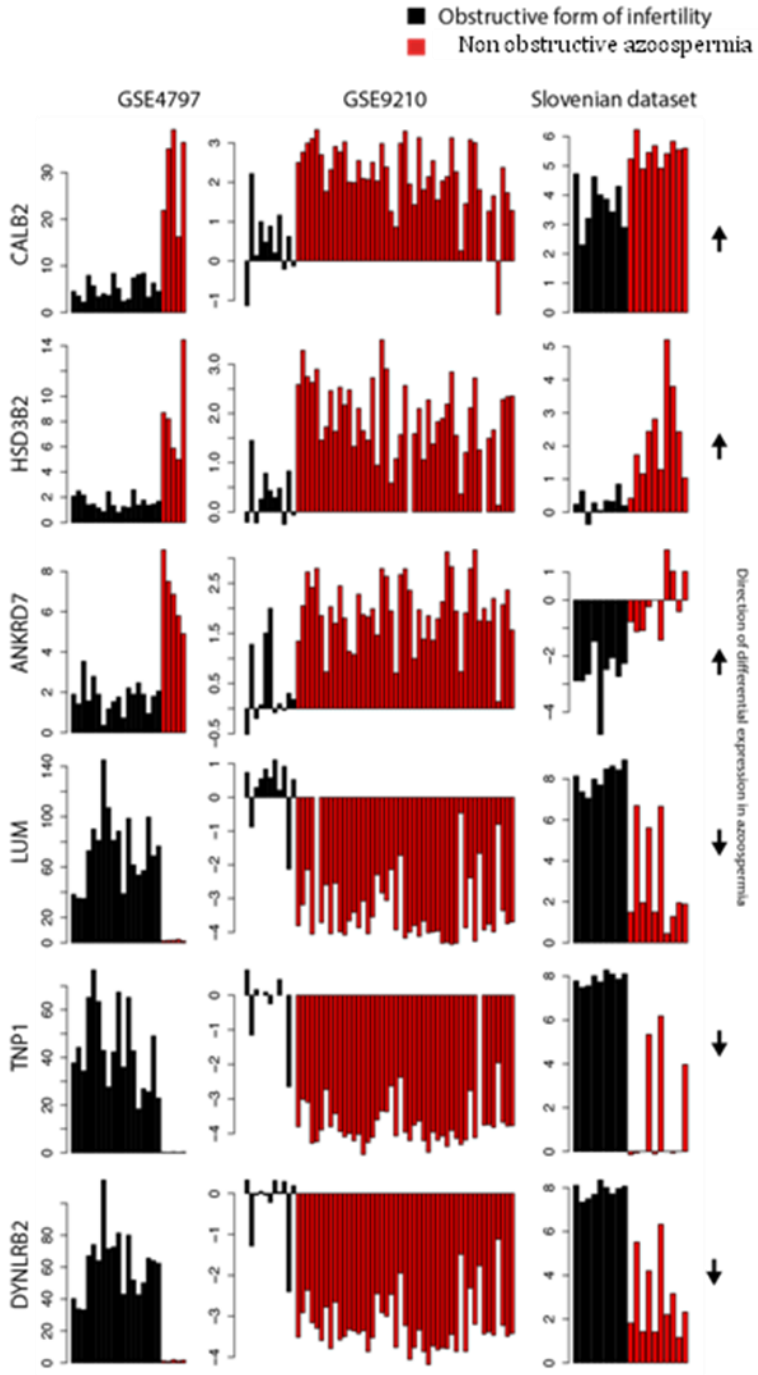

Figure 18: Concordance of expression profiling results for top 6 genes generated by the meta-analysis of three gene expression datasets
Slika 18: Ujemanje profilov izražanja 6 najvišje uvrščenih genov v meta-analizi globalnega merjenja sprememb v treh vključenih študijah

Table 13: Results of meta-analyses from three studies of global gene expression profiling in male infertility
All genes listed in the table, have reached meta-analysis PFP values below 0.0001. Top 10 most highly differentially expressed genes are listed in the upregulated and downregulated set. Genes are sorted according to RankProd score, which is a measure of rank consistency across different studies (larger numbers represent more consistent differential expression).
Preglednica 13: Rezultati meta-analize treh študij izražanja genov pri moški neplodnosti
V tabeli je naštetih po 10 najvišje uvrščenih genov z zvišanim in znižanim izražanjem. Geni so razvrščeni po RankProd vrednosti, ki je mera za konsistenost uvrščenosti gena med različnimi študijami (manjše vrednosti odsevajo konsistenco uvrščenosti različnega izražanja).

TOP 10 UPREGULATED META-ANALYSIS GENES					
RefSeq accession	Entrez gene	Gene symbol	Gene name	RankProd score	Direction
NM_001740	794	CALB2	calbindin 2	57.8	↑
NM_000198	3284	HSD3B2	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	92.1	↑
NM_002345	4060	LUM	lumican	92.5	↑
NM_002889	5919	RARRES2	retinoic acid receptor responder (tazarotene induced) 2	99.9	↑
NM_004673	9068	ANGPTL1	angiopoietin-like 1	104.1	↑
NM_032623	84709	C4orf49	chromosome 4 open reading frame 49	112.3	↑
NM_000204	3426	CFI	complement factor I	121.4	↑
NM_005410	6414	SEPP1	selenoprotein P, plasma, 1	135.4	↑
NM_002178	3489	IGFBP6	insulin-like growth factor binding protein 6	135.5	↑
NM_001734	716	C1S	complement component 1, s subcomponent	146.6	↑
TOP 10 DOWNREGULATED META-ANALYSIS GENES					
RefSeq accession	Entrez gene	Gene symbol	Gene name	RankProd score	Direction
NM_019644	56311	ANKRD7	ankyrin repeat domain 7	3.5	↓
NM_003284	7141	TNP1	transition protein 1 (during histone to protamine replacement)	5.3	↓
NM_130897	83657	DYNLRB2	dynein, light chain, roadblock-type 2	13.6	↓
NM_031916	83853	ROPN1L	rhophilin associated tail protein 1-like	14.6	↓
NM_003886	8852	AKAP4	A kinase (PRKA) anchor protein 4	17.6	↓
NM_012189	26256	CABYR	calcium binding tyrosine-(Y)-phosphorylation regulated	18.4	↓
NM_004362	1047	CLGN	calmegin	19.2	↓
NM_021114	6691	SPINK2	serine peptidase inhibitor, Kazal type 2 (acrosin-trypsin inhibitor)	20.2	↓
NM_003296	7180	CRISP2	cysteine-rich secretory protein 2	25.4	↓
NM_053006	23617	TSSK2	testis-specific serine kinase 2	26.5	↓

4.3.1 Gene set enrichment analyses

To gain more insight into key processes that may possibly explain functional differences among the testicular samples from severely impaired and normal spermatogenesis types, we carried out functional annotation analysis of the top differentially expressed genes, attaining adjusted p-values values below 0.01 and using hypergeometric test, based on

genes' relation to GeneOntology and KEGG terms. The analysis revealed an enrichment of Gene Ontology (GO) terms related to mitotic and meiotic cell cycle, spermatid differentiation and development, microtubule organization and germ cell development (Table 14).

Table 14: Gene set enrichment analysis of down-regulated genes using GeneOntology functional annotations (biological process – BP branch)
Table 14: Obogatitvena analiza genov z znižanim izražanjem v meta-analizi s funkcijskimi antoacijami ontologije GeneOntology (biološki procesi)

Term	P-value*
spermatogenesis	2.5E-13
nuclear division	2.6E-12
mitosis	2.6E-12
microtubule cytoskeleton organization	1.4E-09
reproductive process in a multicellular organism	6.9E-08
chromosome organization	4.5E-07
modification-dependent protein catabolic process	1.4E-06
DNA repair	1.1E-05
nuclear mRNA splicing, via spliceosome	4.8E-05
DNA replication	8.3E-05
regulation of ubiquitin-protein ligase activity	3.0E-04
chromatin organization	3.6E-04
positive regulation of ubiquitin-protein ligase activity	5.4E-04
spindle organization	8.9E-04

* Adjusted for multiple testing according to Benjamini-Hochberg
* Popravljene p vrednosti po Benjamini-Hochberg metodi

Table 15: Gene set enrichment analysis of up-regulated genes using GeneOntology functional annotations (biological process – BP branch)
Table 15: Obogatitvena analiza genov z zvišanim izražanjem v meta-analizi s funkcijskimi antoacijami ontologije GeneOntology (biološki procesi)

Gene Ontology biological process term	P-value*
oxidation reduction	3.20E-14
response to organic substance	8.80E-09
steroid biosynthetic process	2.80E-08
regulation of cell death	1.10E-05
lipid biosynthetic process	9.30E-06
regulation of apoptosis	1.10E-05
sterol metabolic process	2.40E-05
response to wounding	3.90E-05
cellular hormone metabolic process	1.80E-04
blood vessel development	2.60E-04
cholesterol biosynthetic process	2.50E-04
regulation of cell proliferation	3.60E-04
homeostatic process	3.60E-04
vasculature development	4.40E-04
regulation of cell motion	9.20E-04

* Adjusted for multiple testing according to Benjamini-Hochberg
* Popravljene p vrednosti po Benjamini-Hochberg metodi

5 DISSCUSION

Progress in understanding the pathological mechanisms underlying male infertility has been greatly hampered by the complex nature of the disorder. Multiple lines of evidence suggest that genetic factors play an important role in fertility. The main objective of this thesis was to identify genes that play a role in the etiology of male infertility. To achieve this goal, we studied the genetic variability in circadian rhythm genes and gene expression profiles in the human testis. We provide evidence that genetic variability in circadian rhythm genes is associated with male infertility and we identified several differentially expressed genes from testis transcriptome between patients with severely impaired and normal spermatogenesis, and consequently confirmed our hypothesis.

For hypothesis based approach we performed a genetic association study of polymorphic sites in CLOCK and ARNTL genes. We found evidence of an association between male infertility and gene variants of the CLOCK gene in a sample of 961 men (Hodžić et al., 2013). We found a statistically significant difference in the allelic distribution of three SNPs in the CLOCK gene, rs11932595, rs6811520 and rs6850524. A statistically significant difference in haplotype distributions was also confirmed at the CLOCK gene locus when comparing the frequencies of haplotypes TCAG, CCGG and CGGG between the infertile patients and fertile controls. The SNPs interrogated in the ARNTL gene, rs3789327, rs1481892 and rs4757144 did not show significant associations of genotype or allelic distribution between the two groups. According to significant association between CLOCK gene SNPs with male infertility, we also checked the expression of CLOCK and ARNTL genes on our microarray experiment. Gene expression of those two genes showed altered expression levels in the patients with severely impaired spermatogenesis.

Circadian rhythm genes provide promising candidate genes for studies of genetic contributions to male infertility. Circadian rhythms manage a large variety of physiological and metabolic functions and any disruption of these rhythms may affect human health. Although contribution of genetic variations of circadian rhythm genes in infertility in males has not been investigated so far, a few studies indicated the role of the circadian system in male fertility. Recently, Zhang et al. (2012) reported that CLOCK gene SNPs are associated with altered semen quality in idiopathic infertile males (Zhang et al., 2012). Also, it has been shown that night shift workers have an increased risk of infertility (El-Helaly et al., 2010) and that infertile night shift workers have increased serotonin levels and decreased sperm quality compared to fertile night shift workers (Ortiz et al., 2010). Serotonin is namely another component of the circadian system, potentially playing a role in human fertility (Segal et al., 1975) and it is necessary for the development of normal spermatogenesis in rats (Aragon et al., 2005). The circadian system influences testosterone production in humans, showing morning peaks and evening decrease (Cooke et al., 1993; Teo et al., 2011; Bribiescas and Hill, 2010). Moreover, serum levels of sex steroid hormones have been associated with genetic variants in the circadian rhythm genes (Chu et al., 2008). More data is available regarding the role of the circadian system in male infertility in animals. In vitro shifted light-dark cycles influenced the rhythm of sperm release in gypsy moth (Giebultowicz et al., 1989). The study showed that the testis-seminal ducts complex has circadian pacemaker which controls the rhythm of sperm movement in these insects. The fact that clock genes are rhythmically and autonomously expressed in

testes and seminal vesicles was also confirmed in the flies models (Beaver et al., 2002). Beaver et al. (2002) showed that mutation of circadian genes influences reproductive fitness in Drosophila melanogaster. Clock-mutant males produced significantly fewer progeny, had lower number of eggs laid per couple and higher number of unfertilized eggs compared with normal production. Also, clock-mutant males released smaller quantities of sperm. In mice, CLOCK mutant males showed reduced fertility (Dolatshad et al., 2006), whereas ARNTL knock-out mice showed reduced sperm counts (Alvarez et al., 2008). Studies of ARNTL knock-out mice also showed deficiencies in steroidogenesis, namely, male ARNTL KO mice had altered levels of reproductive hormones, indicating a defect in testicular Leydig cells. On the other hand, there is evidence that reproductive hormones directly influence the circadian system (Hagenauer and Lee, 2011); dysregulation of the either axis could therefore contribute to reduced fertility.

In addition to its direct effects, the indirect effect of the circadian system on many physiological processes might possibly influence the male reproductive function. For example, the genetic variability in the CLOCK gene has been associated with increased weight and obesity (Sookoian et al., 2008), and loss of the ARNTL gene functions was shown to result in the development of metabolic syndrome in knock-out rats (Shimba et al., 2011). Additionally, altered expression in the circadian rhythm genes has been shown to occur in obese males (Tahira et al., 2011). The negative effect of obesity on male reproductive function has been substantiated in numerous studies (Cabler et al., 2010; Du Plessis et al., 2010; Hammoud et al., 2006).

In women, genetic variability in the circadian rhythm genes ARNTL and NPAS2 has been suggested to contribute to fertility and seasonality, whereas the genetic variability in the ARNTL gene has been related to a higher number of pregnancies and also to a higher number of miscarriages; polymorphisms in the Npas2 gene have been associated with a decreased number of miscarriages (Kovanen et al., 2010).

Our results provide evidence of novel association between genetic variability in the CLOCK gene and male infertility, and consequently imply the role of circadian timing system in human reproduction. Although results confirmed the importance of circadian mechanisms in male infertility, genetic polymorphisms in the CLOCK gene are themselves not sufficiently strong risk factors to be used as diagnostic biomarkers. Further confirmation and mechanistic investigation of circadian system in male infertility is warranted.

For hypothesis free approach, we performed global gene expression profiling on testis samples belonging to patients with severely impaired and normal spermatogenesis with focus on possible molecular changes in Sertoli and Leydig cells.

Human spermatogenesis is a complex process and for a proper functioning it requires the finely tuned regulation of involved genes. Therefore, alternations in gene expression may represent one of the major molecular mechanisms underlying impaired spermatogenesis. Previously, many microarray studies have investigated changes in global gene expression that are associated with pathological testes (Feig et al., 2006; Ellis et al., 2007; Spiess et al., 2007; Okada et al., 2008). Although these microarray studies have provided useful

insights into the testis transcriptome, they were limited in depth in that they only examined expression changes on the germ cell level and did not have insight into the complexity of the testis transcriptome referring to the somatic cell level. Also, previous studies showed inconsistent results, and therefore, information of the groups of genes which proved to have a reliable role in the etiology of male infertility is still missing.

With global gene expression profiling we have identified a number of genes with altered expression levels in samples with severely impaired spermatogenesis. With a strict statistical criterion (adj. $p < 0.00001$) we identified more than 6000 differentially expressed genes. Of these, 4362 were down-regulated and 471 genes had at least 4-fold down-regulated expression in the samples with severely impaired spermatogenesis compared to samples with normal spermatogenesis.

To gain more insight into the functional profile of alterations occurring among the two groups, we performed gene set characterization of the top differentially expressed genes. The analysis revealed an enrichment of Gene Ontology (GO) terms related to mitotic and meiotic cell cycle, spermatid differentiation and development, microtubule organization and germ cell development. KEGG pathway analysis showed an enrichment of functional categories including protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis, lysosome, phagosome and Escherichia coli infection which correspond to inflammation and autoimmune activity. It is known that infections and inflammations of the male urogenital tract are an important etiological factor of male infertility. Endoplasmic reticulum dysfunction is an important feature of immune regulation. ER stress has been already linked to obesity and CFTR expression, by decreasing CFTR expression at the transcriptional, translational and maturational levels (Ozcan et al., 2004; Rab et al., 2006). The role of obesity and CFTR in male infertility has been already elucidated (Cabler et al., 2010, Dörk et al., 1997). Phagosome and lysosome are the key components of the innate immune response. Also, an important role in the control of immune response against pathogens emanating from the ductal system is played by Sertoli cells (Riccioli et al., 2006). Many of the negative effects of inflammation on spermatogenesis may be attributed to the elevated production of inflammation-associated gene products which result in the impaired Sertoli cell function and their ability to provide proper conditions for germ cell development and survival (Hedger, 2011). Escherichia coli infection is a common cause of the epididymitis and epididymal tubal blockage, which by impeding movement of spermatozoa through ductal system decreases fertility (Pellati et al., 2008; Holmes et al., 1979; Kang et al., 2007). Anyhow, the underlying mechanisms by which pathogens impair spermatogenesis are still unclear. Studies on animal models indicate that underlying reason may be the silent continuation of inflammation that affects fertility by germ cell loss or alternatively duct obstruction and possible implicate E. coli infections in idiopathic infertility causes (Ludvig et al., 2002).

To further investigate molecular changes occurring in impaired spermatogenesis and to define how differentially expressed genes interact with each others in specific pathways, we also performed IPA analysis. IPA analysis revealed several gene networks enriched with genes characterized by significant differential expression in our global expression profiling study. Among the most prominent networks, noticeable are the networks related to cell cycle, cell signalling, cellular organization, urological development and function as

well as DNA replication, recombination and repair. Interestingly, the three top ranked networks consist of genes that almost all interact with Aurora kinase C gene (AURKC). AURKC is cell cycle regulatory serine-threonine kinase, essential for the successful accomplishment of mitotic cell division and which has been previously associated with infertility (Khelifa et al. 2011). The mutation of the AURKC gene has been reported to cause meiosis I arrest and large-headed, multiflagellar polyploid spermatozoa (Dieterich et al., 2009; Khelifa et al., 2012). Another pathway through network genes that we can relate with infertility is the CFTR gene related pathway. It has been shown that networks also contain genes that are associated with CFTR gene. The role of the CFTR gene in male infertility has been previously demonstrated (Dörk et al., 1997). Studies showed that mutations of the CFTR gene may result not only in a Congenital bilateral absence of vas deferens (CBAVD) but also in different forms of male infertility such as azoospermia, teratospermia and oligoasthenospermia (Chen et al., 2012). In the second top ranked network, IPA linked our genes due to possible interaction with ubiquitin and NFkB complex, whereas genes from the third network, IPA linked to ERK12, TSH, MHC class II complex, Rar, PTPase and proinsulin. Ubiquitin is expressed by epididymal epithelium and studies showed that defective sperm is strongly ubiquitinated on the surface (Sutovsky et al., 2001). Nuclear factor kappa B (NFkB) is a complex of transcription factors found in all cell types and involved in the activation of many genes in response to infections and inflammation (Brasier, 2006). NFkB is also connected with the ubiquitination process. Exposure of cells to a variety of extracellular stimuli leads to the rapid ubiquitination and proteolytic degradation of IkappaB which is NF-kappaB inhibitory protein, allowing it to regulate gene transcription (Karin and Ben-Neriah, 2000). The association of genes with ubiquitin and NFkB suggest that altered gene expression may be the result of infections or inflammations. TSH is a thyroid stimulating hormone (TSH), and increased levels of thyroid hormones have negative effect on erectile function and sperm parameters, including sperm count, morphology, and motility (Nikoobakht et al., 2012). Over-expression of insulin in Leydig cells was associated with germ cell loss (Shirneshan at al., 2008). The study of Chung et al. showed that Rar inhibits spermatogenesis in mice (Chung et al., 2011). The role of genes located within the major histocompatibility complex (MHC) had been already investigated for the regulation of reproductive processes in animals and humans. Genes from the three highest ranked networks were all significantly down-regulated, showing complete switch off of the regulated genes in specific pathways.

Also, IPA revealed two significantly upstream regulatory factors that can explain the observed gene expression changes. ADCYAP1 and PAPOLB were inhibited, whereas cycloheximide, fulvestrant and FIGLA were activated. PAPOLB is a testis specific polyA polymerase. ADCYAP1 has been already assumed to affect fertility in females (Isaac and Sherwood, 2008). FIGLA is a transcription factor that plays a key regulatory role in the expression of multiple oocyte specific genes. Cycloheximide affects human sperm function decreasing the acrosome reaction (Naz, 1998). Microarray analyses showed that treatment with fulvestrant changes at least 2-fold the expression of more than 300 genes in rats and causes the inhibition of fluid reabsorption in the efferent ductules, leading to seminiferous tubule atrophy and infertility (Yasuhara et al., 2008).

Our qRT-PCR validation of several genes on independent set of samples confirmed the direction and consistency of expression data shown in our study. All validated genes have

shown down-regulation, while eight genes have shown significant differences in expression levels between samples with severely impaired spermatogenesis compared to samples with normal spermatogenesis.

Because the number of study samples (biological replicates) included in microarray studies is generally much lower than the number of tested variables, testing a large number of variables (genes) on such a small number of study samples results in the excess of false-positive and false-negative detections and may contribute to low reproducibility of results across studies performed by different research groups (Allison et al., 2006). Aiming to attenuate these issues, combining datasets from several experiments investigating the same condition in form of a meta-analysis may increase the statistical power for detecting biologically meaningful genes (Ghosh et al., 2003).

With meta-analyses we confirmed the transcriptional changes observed in our study in terms of direction of gene differential expression and selection of the most significantly expressed genes. Meta-analyses have shown an overlap of 22.4 % significantly up-regulated and 20.7 % down-regulated transcripts consistently across the studies. Among the top up-regulated genes are HSD3B2, RARRES2 and IGFBP6 genes. HSD3B2 gene plays a crucial role in the biosynthesis of all classes of hormonal steroids. It has been previously suggested that increased HSD3B2 transcripts are associated with spermatogenic failure. Study have shown that HSD3B2 transcripts levels were significantly increased in azoospermic patients, particularly in men with Sertoli cell only syndrome (Lin et al., 2008). One of the roles of RARRES2 is that of an adipokine and this gene has been already found to be up-regulated in endometriosis (Zafrakas et al., 2008). IGFBP6 over expression was previously detected in the carcinoma in situ cells, which is a precursor for testicular germ cell tumours (Hoei-Hansen et al., 2004). Expectedly, among the top down-regulated genes across the studies dominated testis and sperm specific genes.

Also, gene enrichment analysis of meta-analysis genes confirmed our results within an enrichment of down-regulated genes related to pathways associated with spermatogenesis, mitosis, DNA repair and regulation of ubiquitin-protein ligase activity.

Furthermore, gene enrichment analysis of meta-analysis genes found differentially expressed, revealed a striking up-regulation of genes related to pathways associated with oxidation-reduction, response to organic substance, steroid biosynthetic process, response to wounding and regulation of apoptosis. Oxidative stress and apoptosis are clearly involved in the pathogenesis of male infertility. Semen reactive oxygen species levels are generally higher in men with idiopathic infertility while apoptosis may prevent sperm from maturing (Agarwal and Said, 2004). The up-regulation of genes related in response to organic substance confirms the impact of environmental factors on male infertility (Bonde and Giwercman, 1995). Normal steroid biosynthesis is required for spermatogenesis and studies have already demonstrated the interaction of environmental toxicants with steroid receptors in the testis (Saradha and Mathur, 2006; Danzo, 1997, Waring and Harris, 2005). Our results confirmed the importance of genetic mechanisms involved in inflammation processes and highlighted the importance of oxidative stress impact on infertility.

Given the heterogeneous tissue composition of the testis and the substantial functions of Sertoli and Leydig cells during all phases of spermatogenesis, we have characterized the transcriptome profiles of the testis not only at the whole-organ level, but also at the sub-cellular level. Sertoli and Leydig cells have a well-established role through all phases of the spermatogenesis process (de Kretser et al., 1998). Although the whole testis analysis showed significantly differentially expressed genes, cell type specific analysis revealed no significantly expressed genes in the Sertoli and Leydig cells between severely impaired and normal spermatogenesis samples.

MDS analysis of all samples resulted in the clear separation between severely impaired and normal spermatogenesis types. However, there was no clear distribution related to the proportion of the Sertoli and Leydig cells in tissue samples. Considering apparent changes observed in all tissues, transcripts from germ cells were either very dominant or we could not detect transcriptomal changes in the Sertoli and Leydig cells. The cause could originate from local histological heterogeneity across different parts of the testis in patients with severely impaired spermatogenesis. The distribution of cells could differ in different parts of the testis and it is possible that the percentages that we measured did not sufficiently represent cellular proportions across the whole testis.

With the transcriptomic study we identified several genes that are implicated in the impaired spermatogenesis and which contributed to the understanding of gene expression involved in pathogenesis of male infertility. We have also shown several new genetics mechanisms that could be more accurately studied in the future.

6 CONCLUSION

With the genetic association study we found a statistically significant difference in the genotype distribution between the infertile and fertile men of the three SNPs in the CLOCK gene: rs11932595 ($p=6\cdot10^{-5}$, OR=1.9 with 95 % CI 1.4-2.7), rs6811520 ($p=2\cdot10^{-3}$, OR=1.7 with 95 % CI 1.2-2.2) and rs6850524 (p=0.01, OR=1.4 with 95 % CI 1.1-1.9). A statistically significant difference in haplotype distributions was also confirmed at the CLOCK gene locus when comparing the frequencies of haplotypes TCAG ($p=8\cdot10^{-5}$, simulated p value after 10.000 permutations was $5\cdot10^{-5}$), CCGG ($p=8\cdot10^{-3}$, simulated p value was $8\cdot10^{-3}$) and CGGG (p=0.04, simulated p value equaled to 0.04) between the infertile patients and fertile controls.

We did not find significant differences in the genotype or allelic distributions of the investigated SNPs for ARNTL gene between the two groups. Accordingly, we did not find any significant difference when comparing the frequencies of the 6 most frequent haplotypes for the 4 analyzed SNPs in the ARNTL gene in the study and control groups.
Anyhow, we confirmed the hypothesis that circadian rhythm genes are attractive candidate genes for male infertility and that genetic variability in the circadian rhythm genes might be associated with male infertility. Consequently, we implied the role of the circadian timing system in human reproduction.

With the genome wide expression study we identified a panel of genes with potential roles in normal and severely impaired spermatogenesis. Most of the detected differentially expressed genes were down-regulated in patients with severely impaired spermatogenesis. We observed significant enrichment of genes related to mitotic and meiotic cell cycle, spermatid differentiation and development, microtubule organization and germ cell development among the top differentially expressed genes. We observed enrichment of functional categories including protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis, lysosome, phagosome and Escherichia coli infection which correspond to inflammation and autoimmune activity. Analysis also revealed the inhibition of upstream regulatory factors ADCYAP1 and PAPOLB, and the activation of cycloheximide, fulvestrant and FIGLA. We confirmed the hypothesis that with the transcriptomic study we could identify new mechanisms underlying pathogenesis of infertility.

By performing meta-analysis we found a high concordance between the results of the three studies in terms of direction of genes differential expression as well as the selection of most significantly differentially expressed genes. We also revealed new potentially interesting up-regulated genes in samples with severely impaired spermatogenesis. Among the top up-regulated genes were HSD3B2, RARRES2 and IGFBP6. Meta-analysis of genes found differentially expressed revealed a striking up-regulation of genes related to pathways associated with oxidation-reduction, response to organic substance, steroid biosynthetic process, response to wounding, and regulation of apoptosis. With the integration of our results with previous studies we confirmed the hypothesis that we can improve the reliability of the data.

Our results confirmed the importance of genetic mechanisms involved in inflammation processes and highlighted the importance of oxidative stress impact on infertility.

Nevertheless, we did not detect significantly differentially expressed genes in the Sertoli and Leydig cells between the patients with severely impaired and normal spermatogenesis. We rejected the hypothesis that more altered genes in patients with severely impaired spermatogenesis are located in the Sertoli and Leydig cells.

7 SUMMARY (POVZETEK)

7.1 SUMMARY

Male infertility is a complex disorder and despite extensive research the etiology in most cases remains unknown. There is increasing evidence that a significant percentage of male infertility is caused by the genetic factor and therefore a better understanding of molecular mechanisms underlying infertility may hold promise in solving unexplained cases. The main objective of this thesis was to identify genes that play a role in the etiology of idiopathic male infertility. To achieve this goal, we used experimental data derived from genetic association and transcriptomic studies.

There is a growing body of evidence that circadian rhythms have a major role in maintaining homeostasis and proper body function, including reproductive capacity. This is highlighted most obviously in animal models. Studies on fruit fly Drosophila, gypsy month and mice showed that disturbances of circadian system and mutations in the CLOCK and ARNTL genes are related to infertility. The circadian system and the main circadian system genes, CLOCK and ARNTL, are highly conserved from flights to humans. Anyhow, despite data on the influence of the circadian system in animal reproduction, there is very limited knowledge regarding the role of the circadian system in infertility in humans. Only a few studies have investigated the role of the circadian system in male fertility. Shift workers have been reported to have an increased risk of infertility and infertile night shift workers have increased serotonin levels and decreased sperm quality compared to fertile night shift workers. It is also known that variations in the testosterone levels are under the influence of the circadian system.

We therefore hypothesized that genetic variability in circadian rhythm genes is associated with male infertility.

We performed a retrospective case-control genetic association study of polymorphic sites in CLOCK and ARNTL genes on a population of patients with male infertility in comparison with a fertile male control population. Our study group consisted of 517 patients with idiopathic infertility and a control group of 444 fertile men. Eight tagging single nucleotide polymorphisms (SNPs) were chosen from both genes. We based the selection of SNPs on the known genetic linkage in both genes and the chosen SNPs represent the set of the most representative tagSNPs for ARNTL and CLOCK genes. SNPs genotyping was carried out by real time PCR method.

We found statistically significant difference in the genotype distribution in the CLOCK gene: rs11932595 ($p=6\cdot10^{-5}$), rs6811520 ($p=2\cdot10^{-3}$) and rs6850524 ($p=0.01$). Further analyses of haplotypes were consistent with genotyping results.

A lot of information of the etiology, pathogenesis and homeostatic mechanisms underlying infertility has been recently obtained from ‘omic’ studies. Global gene expression profiling on pathological testis samples identified a group of genes potentially associated with infertility, but results were not consistent and were difficult to interpret. Also, the main focus was on transcriptional changes in germ cells, whereby possible effects of altered

expression in somatic cells have been neglected. Human spermatogenesis is a complex process and beside germ cells it is dependent on two other specialized cell types, Sertoli and Leydig cells. Sertoli and Leydig cells have irreplaceable functions through all phases of spermatogenesis.

We hypothesized that with a transcriptomic study we could identify new mechanisms underlying the pathogenesis of infertility and that with additional focus on Sertoli and Leydig cells we could find novel altered genes in patients with severely impaired spermatogenesis.

We performed global gene expression profiling on testis samples belonging to patients with severely impaired and normal spermatogenesis and cell type-specific analysis of microarrays to get more insight on possible molecular changes in Sertoli and Leydig cells during impaired spermatogenesis. Furthermore, we performed meta-analyses of gene expression data across available transcriptomic studies.

The study included 20 samples (10 showing obstructive azoospermia and 10 samples showing non obstructive azoospermia). The measure of expression was performed on isolated RNA from biopsy samples of patients with obstructive and non obstructive azoospermia using Agilent Whole Human Genome 4x44 microarrays. Differential gene expression was measured in relation to the Agilent's Universal Human Reference RNA and each experimental sample was hybridized against this common reference sample.

We identified more than 6000 differentially expressed genes. Most of the differentially expressed genes were down regulated in patients with severely impaired spermatogenesis. Among these, significant enrichment of genes related to mitotic and meiotic cell cycle, spermatid differentiation and development, microtubule organization and germ cell development as well as functional categories, including protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis, lysosome, phagosome, and Escherichia coli infection which correspond to inflammation, and autoimmune activity were found. We validated several differentially expressed genes from our experiment by qRT-PCR on fifteen independent samples and confirmed the direction and consistency of expression data from our study. Our results confirmed the importance of genetic mechanisms involved in inflammation processes.

With meta-analysis of our study and the existing transcriptomic studies, we confirmed the transcriptional changes observed in our study and revealed new genes related to pathways associated with oxidation-reduction, response to organic substance, steroid biosynthetic process, response to wounding and regulation of apoptosis.

The cell type specific analysis revealed no significantly expressed genes in the Sertoli and Leydig cells between severely impaired and normal spermatogenesis patients.

In conclusion, our results provide evidence of novel association between circadian genes and male infertility and imply the role of the circadian rhythm system in human reproduction. The results of the transcriptome study confirmed the importance of genetic mechanisms involved in inflammation processes and highlighted the importance of

oxidative stress impact on infertility, and contributed to the understanding of gene expression involved in the pathogenesis of male infertility.

7.2 POVZETEK

Neplodnost prizadene približno 9 % odrasle populacije (Boivin in sod., 2007), pri čemer je približno polovica primerov povezana z dejvnikom neplodnosti pri moškem (Agarwal in Said, 2003). Pri 30 % neplodnih moških etiologije še vedno ne odkrijemo (idiopatska moška neplodnost), predvsem zaradi slabega razumevanja molekularnih mehanizmov ki igrajo vlogo pri moški neplodnosti (Jungwirth in sod., 2012; Matzuk in Lamb, 2002). Ocenjuje se, da so genetski dejavniki pomembno udeleženi v patogenezi pri 50 % moških z idiopatsko neplodnostjo (Hwang in sod., 2010).

Genetski dejavniki vpleteni v neplodnost so na vseh ravneh genetskih informacij, od kromosomov, prek genov do sprememb posameznih nukleotidov (O' Flynn in sod., 2010). Ob tem do sedaj poznani specifični genetski dejavniki, povezani z neplodnostjo, pojasnijo etiologijo največ 50% dedne komponente idiopatske moške neplodnosti, kar kaže na potrebo po več raziskavah na tem področju. Glavni cilj disertacije je bil odkriti gene, ki igrajo vlogo v etiologiji idiopatske moške neplodnosti. Z namenom dosega tega cilja smo uporabili eksperimentalne pristope genetskih asociacijskih in transkriptomskih študij.

V smislu prispevka genetske variabilnosti smo izbrali genske različice v genih, vključenih v regulacijo cirkadianega ritma. Cirkadiani ritmi se pojavljajo v organizmu s približno 24 urno periodo. Tvorijo jih vedenjski in fiziološki procesi, ki omogočajo organizmu, da predvidi okoljske spremembe in se pravočasno na njih pripravi. Cirkadiani ritmi igrajo ključno vlogo pri zdrževanju homeostaze in normalnega delovanja telesnih funkcij, vključno z reproduktivnimi funkcijami (Kennaway in sod., 2012). Raziskave na živalskih modelih so pokazale, da spremembe v cirkadianem ritmu vplivajo na normalno reproduktivno sposobnost. Laboratorijske študije so pokazale, da motnja v rimičnosti ciklov svetlobe vpliva na ritem sproščanja sperme pri insektih (Giebultowicz in sod., 1989). V isti študiji so pokazali, da imajo testikularno-semenski kanali lasten cirkadiani spodbujevalnik, ki nadzoruje ritem pomikanja spermijev. Spontana ritmična aktivnost genov cirkadianega sistema je bila podobno pokazana v testisu vinske mušice (Beaver in sod., 2002). Mutacije CLOCK genov pri Drosophili melanogaster vplivajo na reproduktivno zmožnost in moško neplodnost. CLOCK – mutirani samci proizvedejo manj potomcev in tvorijo manjšo količino sperme (Beaver in sod., 2002). Cirkadiani protein ARNTL je pri miših potreben za plodnost in zadostno tvorbo testosterona (Alvarez in sod., 2008). Študije so pokazale nepravilnosti pri steroidogenezi. Samci ARNTL knock-out miši so imeli nizke koncentracije testosterona in visoke koncentracije luteinizirajočega hormona, kar kaže na nepravilnosti delovanja Leydigovih celic v testisih.

Primarni cirkadiani geni CLOCK in ARNTL so visoko filogenetsko ohranjeni med vrstami (Vansteensel in sod., 2008). Molekularni elementi genov cirkadianega sistema sestavljajo pozitivne in negativne transkripcijsko-translacijske regulatorne zanke večih genov (Okamura in sod., 2003). Gena CLOCK in ARNTL predstavljata osrednje vozlišče v tem omrežju in tvorita pozitivno povratno zanko s heterodimerizacijo in spodbujanjem prepisovanja drugih cirkadianih genov (Zhang in sod., 2004). Nastale beljakovine oblikujejo negativno regulatorno zanko in inhibirajo trankripcijsko stimulacijo genov CLOCK in ARNTL (Reppert in Weaver, 2002).

Kljub veliko podatkov o vplivu cirkadianega sistema na reprodukcijo pri živalih, je zelo malo znanja o vplivu cirkadianega sistema na neplodnost pri človeku. Le v nekaj študijah so raziskovali vlogo cirkadianega sistema pri moški neplodnosti. V zadnjem času so Zhang in sod. (2012) poročali da so polimorfizmi v CLOCK genu povezani s spremenjeno kvaliteto spermijev pri moških z idiopatsko neplodnostjo (Zhang in sod., 2012). Prav tako je bilo pokazano, da imajo delavci v nočni izmeni večje tveganje za neplodnost (El-Helaly in sod., 2010) in da imajo neplodni delavci v nočnih izmenah povišane nivoje serotonina in slabše semenske parametre v primerjavi s plodnimi delavci v nočni izmeni (Ortiz in sod., 2010). Serotonin je drugi sestavni del cirkadianega sistema, ki potencialno igra vlogo v plodnosti pri človeku (Segal in sod., 1975) in je nujen za normalno spermatogenezo pri podganah (Aragon in sod., 2005). Cirkadiani sistem vpliva tudi na izločanje testosterona pri človeku, in kaže jutranje vrhove in znižano sproščanje v večernem času (Cooke in sod., 1993; Teo in sod., 2011; Bribiescas in Hill, 2010). Nadalje so serumski nivoji spolnih steroidnih hormonov povezani z genetskimi različicami v cirkadianih genih (Chu in sod., 2008).

Zaradi vpliva bioritmičnih genov na neplodnost pri živalih, smo predvidevali, da so bioritmični geni vpleteni v moško reprodukcijo in da je genetska variabilnost bioritmičnih genov povezana z moško neplodnostjo.

Zasnovali smo genetsko asociacijsko raziskavo, v kateri smo primerjali pogostnost genotipov v bioritmičnih genih med neplodnimi moškimi in plodnimi kontrolami. V genih ARNTL in CLOCK je bila predhodno opisana variabilnost, predvsem v obliki enoonukleotidnih polimorfizmov (SNP). Zaradi številčnosti SNPov v regiji teh dveh genov smo najprej opravili selekcijo SNPov in jih izbrali glede na strukturo genetske vezave v teh dveh genih. Pri tem smo uporabili podatke iz baze HapMap. Izbrali smo SNPe, ki zastopajo največ možnih bližnjih SNPov (ti. tagSNPe) in na ta način pokrili čimvečji delež variabilnosti v genu s preiskovanjem čimmanjšega števila SNPov. Za zbrane SNP in analizo smo vporabili PCR v realnem času.

Odkrili smo statistično pomembno razliko v pogostosti genotipov CLOCK gena pri polimorfizmih rs11932595 ($p=6\cdot10^{-5}$), rs6811520 ($p=2\cdot10^{-3}$) in rs6850524 ($p=0.01$). Rezultati nadaljnjih analiz haplotipov v izbranih genih so podpirali rezultate genotipizacije posameznih polimorfizmov.

Naši rezultati dajejo podpore nove povezave med genetskimi različicami v CLOCK genu in moško neplodnostjo, ter posledično nakazujejo vlogo cirkadianih ritmov pri reprodukciji človeka. Kljub temu da so rezultati pokazali na potencialen pomen cirkadianih ritmov pri reprodukciji človeka, pa preiskane genetske različice v CLOCK genu same po sebi niso dovolj močni dejavniki tveganja, da bi jih uporabili kot samostojne genetske biooznačevalce. V ta namen bodo potrebne nadaljnje potrditvene in mehanistične študije vpliva cirkadianega sistema pri moški neplodnosti.

Velik obseg informacij o etiologiji, patogenezi in homeostatskih mehanizmih pri neplodnosti so v zadnjem času ponudile transkirptomske, proteomske in metabolomske študije (von Kopylow in sod., 2010; Johnston in sod., 2005; Pilch in Mann, 2006; Deepinder in sod., 2007). “Omske” tehnologije omogočajo nov vpogled na patogenezo

neplodnosti in so že pokazale velik potencial za razkrivanje vzročnih procesov, ki igrajo ključno vlogo pri neplodnosti. Transkriptomske študije omogočajo sočasno merjenje ekspresije velikega deleža genov v humanem genomu in tako omogočajo celosten vpogled v gensko regulacijo, povezano z molekularno patogenezo in homeostatskimi mehanizmi na nivoju izražanja genov (He in sod., 2006).

Do danes je bilo opravljenih le nekaj študij, pri katerih so uporabili pristop merjenja razlik v genski ekspresiji na globalnem nivoju. V pravi taki študiji so Feig in sodelavci leta 2007 poskušali identificirati gene, ki igrajo vlogo pri posameznih stopnjah razvoja germinalnih celic. Njihovi rezultati so pokazali da se izražanje skupine 1181 genov pomembno spreminja skozi posamezne stopnje razvoja germinalnih celic. Osredotočili so se na tri skupine genov, ki so označevale pre-mejotsko, mejotsko in končno stopnjo diferenciacije, geni v skupinah pa so se med seboj razlikovali po vpletenosti v različne biološke procese, poti in regulatornem vplivu različnih transkripcijskih dejavnikov. Razlike v izražanju germinalno specifičnih genov so bile tudi pokazane v študiji Ellis in sod. (2007), ki so prav tako pokazali, da je zmanjšano prepisovanje germinalno specifičnih genov povezano s povečnim izražanjem vnetnih genov. Z namenom odkrivanja specifičnih patofizioloških procesov in molekularnih poti pri nastanku ne-obstruktivne oblike azoospermije (NOA – angl. non-obstructive azoospermia) so v študiji Okada in sod. 2008 s tehnologijo mikromrež preiskali bioptične vzorce testisov s heterogenimi oblikami moške neplodnosti. Za razliko od študije Feig in sod. (2007), kjer so vzorce ločevali na podlagi Johnsenove vrednosti, so tu poskušali razvrstiti vzorce v podskupine s pomočjo nenadzorovane klasifikacije. S to metodo so uspeli ločiti vzorce NOA v tri razrede na podlagi razlik v izražanju 149 prepisov genov. Prav tako so opredelili ART3 gen kot dejavnik tveganja za NOA.

Na ključno vlogo izražanja genov v somatskih testikularnih celicah pri normalni spermatogenezi so opozorili Speiss in sodelavci leta 2007. Nivoji vsaj 188 prepisov so bili v povezavi z obsežnostjo motenj spermatogeneze in so dosegli najvišje vrednosti pri vzorcih s sindromom Sertolijevih celic. V isti študiji so pokazali tudi obogatenost genov, povezanih z vnetnimi procesi. Tako so povezali pomen funkcije mastocitov, ki sicer igrajo vlogo v širokem naboru bolezni človeku, tudi pri patofiziologiji bolezni testisa.

Pristopi k razvrščanju patoloških slik testisa v ločene razrede na osnovi izražanja genov so v predhodnih študijah dali neenotne rezultate in zato izbor genov, s katerimi bi zanesljivo ločevali posamezne oblike neplodnosti in ga uporabljali kot diagnostični biomarker, še ni znan. Poleg tega so se predhodno opravljene študije osredotočale na transkriptomske spremembe v germinalnih celicah in tako zanemarile možen vpliv spremenjenega izražanja genov v somatskih celicah testisa.

Spermatogeneza pri človeku je dolg in zapleten proces, ki ga nadzoruje sistem hormonskih dejavnikov, modulacija genske ekspresije in medcelične interakcije (Wistuba in sod., 2007). Med spermatogenezo potekajo procesi, ki preoblikujejo osnovne spermatogonije v visoko specializirane spermatozoje in vključujejo procese mitoze, mejoze in intenzivnih morfoloških modifikacij. Čeprav spermatogeneza predstavlja dozorevanje germinalnih celic, pa nanjo vplivajo tudi celice drugih tipov – tako imajo somatske celice osnovno vlogo v vseh stopnjah spermatogeneze.

Sertolijeve celice so visoko specializirane celice testisa, ki so ključne za njegovo formacijo in normalen potek spermatogeneze. Vpletene so tako pri razvoju testisa (Wilhelm in sod., 2007), vključene pa so tudi pri napredovanju germinalnih celic v stopnjo spermatozojev (Griswold, 1998). Nahajajo se v semenskih cevkah in opravljajo številne funkcije. Predstavljajo podporne celice in formirajo krvno-testikularno bariero, ki ovira spermijem vstop v krvni obtok in tako preprečujejo sistemski avtoimunski odziv na spermije (Dym in sod. 1970). Producirajo tudi tekočino semenskih cevk, ki omogoča prehod izločkom testisa v epididimis, zagotavlja prehrano germinalnim celicam in spermatozojem ter omogoča germinalno-germinalno celično in germinalno-Sertoli celično signalizacijo. Poleg tega tudi sintetizirajo in uravnavajo prenos molekul in beljakovin za prehrano, delitev in razvoj germinalnih celic (Jégou, 1993). Sertolijeve celice so vpletene tudi v proces migracije in apoptoze germinalnih celic in sodelujejo pri endokrini regulaciji spermatogenez, saj se preko njih vrši delovanje hormonov FSH in testosterona (Walker in Cheng, 2005).

Leydigove celice v intersticijskem tkivu testisa proizvajajo testosteron in druge androgene, ključne za spermatogenezo in razvoj moških urogenitalnih organov. Testosteron in drugi inrogeni nastajajo kot odziv na luteinizirajoči hormon, ki se veže na receptorje površine Leydigovih celic. Čeprav imajo samo Sertolijeve celice v semenskih kanalčkih receptorje za testosteron, so te Leydigove celice glavne tarče hormonskih signalov za spermatogenezo (Walker in Chang, 2005). Leydigove celice tudi izločajo številne ne-steroidne dejavnike, ki so pomembni za stimulacijo kontrakcije semenskih cevk, beta-endorfin za inhibicijo proliferacije in fukncijskega uravnavanja delovanja Sertolijevih celic, kot tudi EGF, ki je pomemben za regulacijo spermatogeneze (Bostwick in Cheng, 2008).

Na osnovi predstavljenih dejstev smo predvidevali, da bi lahko s transkriptomsko študijo odkrili nove mehanizme, ki sodelujejo pri patogenezi neplodnosti in da bi z osredotočanjem na specifične spremembe v Serolijevih in Leydigovih celicah lahko odkrili nove gene, spremenjene pri pacientih z okvarjeno spermatogenezo. Poleg tega, smo predvidevali da bi lahko z združevanjem naših podatkov z rezultati predhodno opravljenih transkriptomskih študij lahko izbojšali zanesljivost odkritih sprememb v genski ekspresiji.

Opravili smo globalne mertive razlik v ekspresiji na vzorcih testikularnega tkiva pacientov s hudo okvarjeno in normalno spermatogenezo in celično-specifično analizo podatkov eksperimentov na mikromrežah, da bi pridobili boljši vpogled na potencialne molekularne spremembe v Sertolijevih in Leydigovih celicah med moteno spermatogenezo. Nadalje, smo opravili meta-analizo podatkov globalnega izražanja genov iz naše in predhodno opravljenih transkriptomskih študij.

V študijo smo vključili 20 vzorcev (10 vzorcev testikularnega tkiva moških z obstruktivno azoospermijo in 10 vzorcev tkiva moških z ne-obstruktivno azoospermijo). Mertive izražanja genov so bile opravljene na vzorcih RNA osamljenih iz bioptičnih vzrocev pacientov z obstruktivno in ne-obstruktivno azoospermijo z mikromrežami Agilent Whole Human Genome 4x44. Razlike v izražanju genov so bile merjene relativno na referenčni vzorec RNA (Agilent Universal Human Reference RNA) in vsak preiskovani vzorec RNA smo skupaj z refenčnim RNA vzorcem hibridizirali na mikromrežo.

Da bi v večji meri izkoristili informacije dobljene na heterogenih vzorcih testisov, smo opravili celično specifično analizo signifikance na podatkih z mikromrež (cell type-specific significance analysis of microarrays - csSAM) (Shen-Orr in sod., 2010). Pri tem smo uporabili podatke o relativnem deležu posameznih celičnih vrst v testisu posameznega pacienta in s pomočjo statističnega pristopa dekonvolucije matrik iz podatkov ekspresije merjene na celokupnem vzorcu pridobili informacije o razlikah v ekspresiji pri posamezni podskupini celic, zajetih v preiskanem tkivnem vzorcu.

Nivoji izražanja 8 genov, pri katerih smo z metodo mikromrež odkrili največje razlike v izražanju smo preverili tudi s kvantitativno PCR metodo v realnem času. Validacijo smo opravili na novi skupini 15 bioptičnih vzorcev, v kateri smo zajeli 7 neplodnih moških s hudo prizadeto spermatogenezo in 8 neplodnih moških z normalno spermatogenezo.

Z namenom povišanja robustnosti rezultatov in odkrivanja čim bolj konsistentnih sprememb v izražanju genov pri moški neplodnosti, smo vključili tudi podatke predhodno opravljenih študij globalnih genskega izražanja pri moški neplodnosti. Vključili smo študije, katerih podatki so prosto dostopni na javnih repozitorijih podatkov mikromrež: Gene Expression Omnibus (GEO) in ArrayExpress. Vključitveni kriteriji so bili tudi: zasnova eksperimenta sorodna našemu – hibridizacija na mikromrežo z enotno referenčno RNA, preiskovanje vzorcev tkiva dobljenih z biopsijo testisa in vključene oblike moške neplodnosti, primeljive s preiskovanimi v naši študiji. Dve predhodno opravljeni študiji sta ustrezali tem kriterijem, zato smo njihove rezultate vključili tudi v našo študijo ter opravili meta-analizo združenih naših in predhodno opravljenih meritev globalnega izražanja genov pri moški neplodnosti. Meta-analizo smo opravljali s programskim jezikom R 2.9.2 v okolju BioConductor z uporabo programskega paketa RankProd. Neobdelane podatke iz predhodno opravljenih študij smo pridobili iz repozitorija Gene Expression Omnibus (GEO) (Sean in Meltzer, 2007).

Z meritvami globalnih nivojev izražanja genov smo odkrili številne gene s spremenjenim izražanjem pri vzorcih s hudo moteno spermatogenezo. Več kot 6.000 genov je preseglo izbrani rigorozni prag signifikance pod $1 \cdot 10^{-5}$. Od teh genov s spremenjeno ekspresijo smo pri 4362 genih ugotovili znižano izražanje pri moteni spermatogenezi, od teh smo pri 471 našli vsaj 4-kratno znižanje nivojev izražanja pri vzorcih s hudo motnjo v spermatogenezi v primerjavi z vzorci, pri katerih je bila spermatogeneza normalna.

Za boljši vpogled v ključne biološke vloge genov, pri katerih smo ugotavljali spremembe, smo opravili tudi obogatitveno analizo genov z najbolj spremenjenimi nivoji izražanja. Analiza obogatenosti je pokazala visoko statistično pomembno obogatenost genov, ki po podatkih baze GeneOntology pripadajo funkcijskim skupinam povezanim z mitotskim in mejotskim celičnim ciklom, diferenciacijo in razvojem spermatid, organizacijo mikrotubulov in razvojem germinalnih celic. Obogatenost genov, glede na podatke baze o bioloških poteh – KEGG, je pokazala obogatitev funkcijskih skupin, povezanih s premeno beljakovin v endoplazemskem retikulumu, s proteolizo vodeno z ubikvitinskimi modifikacijami, s funkcijami lizosomov in fagosomov, z okužbo z Escherichio coli in z vnetjem ter avtoimunimi procesi. Znano je, da so okužbe in vnetja urološkega trakta pomemben etiološki dejavnik pri nastanku moške neplodnosti. Tudi motnje v funkciji endoplazemskega retikuluma (ER) lahko privedejo do spremenjene regulacije imunskega

odziva. Motnje delovanja ER so bile že predhodno povezane z debelostjo in nivojem izražanja CFTR gena, pri čemer pride do zmanjšanja izražanja CFTR na transkripcijskem, translacijskem in maturacijskem nivoju (Ozcan in sod., 2004; Rab in sod., 2006). Vloga, ki jo imata debelost in CFTR gen pri moški neplodnosti je poznana iz rezultatov predhodnih študij (Cabler in sod., 2010; Dörk in sod., 1997). Tudi funkcija fagosomov in lizosomov je pomemben element delovanja imunskega odziva. Sertolijeve celice so pomemben dejavnik obrambe gostitelja pred patogeni, ki zaidejo v duktalni sistem testisa (Riccioli in sod., 2006). Številni negativni učinki vnetja na spermatogenezo izvirajo iz povečanega izražanja genski produktov, povezanih z vnetjem, kar ima za posledico okvarjeno delovanje Sertolijevih celic in njihovo zmanjšano možnost za nudenje ugodnih pogojev za razvoj in preživetje germinalnih celic (Hedger, 2011). Okužba z Escherichio coli je pogost vzrok za epididimitis in pojav blokado semenskih kanalov, kar ima zaradi omejenega prehoda spermatozojev skozi sistem semenskih kanalov za posledico zmanjšano plodnost (Pellati in sod., 2008; Holmes in sod., 1979; Kang in sod., 2007). Ob tem je poznavanje natančnih mehanizmov s katerimi mikroorganizmi okvarjajo spermatogenezo še vedno omejeno. Študije na živalskih modelih kažejo da je možen vzročni dejavnik tiho nadaljevanje vnetja, ki vpliva na poldnost z manjšanjem populacije germinalnih celic ali pa z obstrukcijo semenskih kanalov in preko tega povezuje okužbo z E. Coli z vzroki za idiopatsko neplodnostjo (Ludvig in sod., 2002).

Z namenom boljše opredelitve molekularnih sprememb, ki se pojavljajo pri moteni spermatogenezi in z namenom odkrivanja medsebojnih interakcij v specifičnih molekularnih poteh, smo opravili tudi funkcijske analize z orodjem IPA (Ingenuity Pathway Analysis). Z analizo IPA smo odkrili številna genska omrežja, v katere so vključeni najbolj različno izraženi geni, glede na rezultate naše študije merjenja globalnega izražanja genov pri moški neplodnosti. Med najbolj izrazito obogatenimi, so omrežja povezana s celičnim ciklom, medcelično signalizacijo, organizacijo celične strukture, razvojem ter funkcijo urotrakta ter replikacijo DNA, rekombinacijo DNA in popravljalnimi mehanizmi DNA. Predvsem izstopa skupna lastnost genov v najvišje obogatenih omrežjih, da so v interakciji s beljakovino, ki jo kodira AURKC (Aurora kinase C) gen. AURKC je regulatorna serinsko-treoninska kinaza, ki je ključna za uspešno zaključitev mitotičnega celičnega cikla in je bila predhodno že povezana z neplodnostjo (Khelifa in sod. 2011). Mutacije gena za AURKC glede na rezultate funkcijskih študij povzročijo zaustavitev celičnega cikla v stopnji mejoze I, spermiji ki nastanejo pri tem pa imajo veliko glavo, imajo nadštevilčne bičke in so multiploidni (Dieterich in sod., 2009; Khelifa in sod., 2012). Druga pomembna pot, v katero so vključeni geni v najbolj obogatenih omrežjih, je pot povezana s funkcijo beljakovinskega produkta gena CFTR. Vloga gena CFTR pri moški neplodnosti je bila že predhodno povezana z moško neplodnostjo na podlagi rezultatov že opravljenih študij. Mutacije v genu CFTR imajo za posledico ne le pojav kongenitalne obojstrenske odsotnosti semenovodov ampak tudi druge oblike neplodnosti, kot je azoospermija, teratospermija in oligoastenospermija (Chen in sod., 2012). V drugem najvišje uvrščenem omrežju je IPA analiza pokazala omrežje genov okrog ubikvitinskega in NFkB kompleksa, medtem ko so geni iz tretjega najvišje uvrščenega omrežja povezani z geni ERK12, TSH, MHC II kompleksom, beljakovino Rar, PTPazo in proinsulinom. Ubikvitin je izražen na epitelnih celicah epididimusa in študije kažejo na visoko stopnjo ubikvitinacije površine nefunkcionalnih spermijev (Sutovsky in sod., 2001). Jederni faktor kapa B (NFkB) je kompleks transkripcijskih faktorjev v vseh celičnih tipih in je vpleten v

aktivacijo številnih genov pri odzivu na okužbe in vnetje (Brasier, 2006). NFkB je prav tako povezan s procesom ubikvitinacije. Izpostavljenost celic številnim izvenceličnim dražljajem vodi v hitro ubikvitinacijo in proteolitično razgradnjo inhibitorja kompleksa NFkB imenovanega I-kapaB, kar faktorju NFkB omogoča regulacijo izražanja genov (Karin in Ben-Neriah, 2000). Povezava odkritih genov s spremenjenim izražanjem pri moteni spermatogenezi kaže na to da je lahko spremenjeno izražanje le-teh posledica okužb in vnetja. TSH je tiroido-stimulirajoči hormon in povečani nivoji hormonov ščitnice imajo zaviralni učinek na erektilno funkcijo in semenske parametre, vključno s številom spermijev, morfologijo in gibljivost (Nikoobakht in sod., 2012). Povišano izražanje insulina v Leydigovih celicah je bilo povezano z izgubo germinalnih celic (Shirneshan at al., 2008). V študiji Chung in sod. so pokazali da Rar zavira spermatogenezo pri mišjih modelih (Chung in sod., 2011). Vloga genov v MHC II kompleksu (MHC) je bila že povezana z regulacijo reproduktivnih procesov pri živalih in ljudeh. Ekspresija skoraj vseh genov iz treh najvišje uvrščenih IPA omrežij je statistično pomembno znižana, kar nakazuje popolno ugaslost teh poti v predstavljenih poteh.

Nadalje smo z IPA analizo odkrili dva statistično pomembno obogatena transkripcijska dejavnika, ki lahko pojasnita spremembe v izražanju genov. Smer spremenjenga izražanja genov je nakazovala profil, ki ustreza inhibiciji dejavnikov ADCYAP1 in PAPOLB in aktivaciji s strani dejavnikov FIGLA, fulvestranta in cikloheksimida. PAPOLB je poliA polimeraza specifična za testikularno tkivo. ADCYAP1 je bila že povezana kot možni dejavnik pri ženski neplodnosti (Isaac in Sherwood, 2008). FIGLA je transkripcijski dejavnik, ki igra ključno uravnalno vlogo v ekspresiji številnih genov specifičnih za oocite. Cikloheksimid vpliva na funkcijo spermijev pri človeku in deluje inhibitorno pri procesu akrosomske reakcije (Naz, 1998). Študije z mikromrežami so pri podganah pokazale da izpostavitev testikularnega tkiva fulvestrantu povzročijo spremembe v izražanju več kot 300 genov in zavira resorpcijo v eferentnih duktulih testisa, kar privede do atrofije tubulov testisa in neplodnosti (Yasuhara in sod., 2008).

Validacija izbranih genov s qRT-PCR na neodvisnem izboru vzorcev je potrdila smer in konsistentnost izmerjenih razlik v izražanju genov, ki smo jih odkrili s preiskovanjem globalnih ekspresijskih razlik na mikromrežah. Vsi geni, izbrani za validacijo so bili manj izraženi v vzorcih bolnikov z motnjami v spermatogenezi, prav tako so bili razlike tudi statistično pomembne.

Ker je število pročevanih vzorcev (bioloških replikatov), vključenih v študije na mikromrežah, v večini primerov bistveno manjše kot število preiskovanih spremenljivk, lahko statistično preverjanje visokega števila spremenljivk na malem vzorcu primerov privede do visokega števila lažno pozitivnih in lažno negativnih rezultatov in tako prispeva k manjši ponovljivosti eksperimenta med različnimi študijami (Allison in sod., 2006). Z namenom zmanjšanja te problematike smo pristopili k združevanju podatkov različnih opravljenih študij, ki so preiskovale različne oblike moške neplodnosti s tehnologijo mikromrež z meta-analizo, kar lahko privede do povečane zanesljivosti odkritih sprememb v izražanju genov in izboljša ponovljivost tako odkritih sprememb v izražanju genov (Ghosh in sod., 2003).

Z meta-analizo smo potrdili spremembe v izražanju genov v naši študiji tako v smislu smeri sprememb v izražanju kot tudi pri določitvi genov z najbolj statistično pomembnimi spremembami v izražanju. Meta-analiza je pokazala prekrivanje 22.4 % genov s statistično pomembnim povišanim izražanjem in 20.7 % genov s statisično pomembno znižanim izražanjem pri vseh vključenih študijah. Med najvišje uvrščenimi geni v meta analizi so bili geni HSD3B2, RARRES2 in IGFBP6 s povišanim izražanjem pri motnjah spermatogeneze. HSD3B2 gen igra ključno vlogo pri biosintezi vseh steroidnih hormonov. Predhodno je bilo pokazano, da so spremembe v njegovem izražanju povezane tudi z motnjami spermatogeneze. Prav tako so predhodne študije pokazale, da so bili nivoji HSD3B2 pomembno povišani pri bolnikih z motnjami v spermatogenezi, posebej izrazito pri moških s sindromom Sertolijevih celic (Lin in sod., 2008). Gen RARRES2 je adipokin, katerega povišano izražanje je bilo pokazano pri endometriozi (Zafrakas in sod., 2008). Povišano izražanje IGFBP6 je bilo prav tako predhodno ugotovoljeno pri prekurzorjih germinalno celičnih tumorjev (Hoei-Hansen in sod., 2004). V skladu s pričakovnji so bili najvišje uvrščeni geni z znižanim izražanjem v meta-analizi testikularno in za spermije specifični geni.

Prav tako je analiza obogatenosti genov, odkritih z meta-analizo potrdila naše rezultate globalne analize razlik v izražanju genov pri moteni spermatogenezi. Med geni z zmanjšanimi nivoji izražanja pri motanjah spermatogeneze smo ugotovili obogatenost genov povezanih s spermatogenezo, mitozo, popravljanjem DNA napak in regulacijo aktivnosti ubikvitinske ligaze.

Nadalje je analiza obogatenosti genov z zvišanim izražanjem v meta-analizi pokazala obogatenost genov v poteh uravnavanja celičnega oksidacijsko-redukcijskega potenciala, pri odgovoru na organske molekule, pri biosintezi steroidov, pri odgovoru na tkivne poškodbe in pri regulaciji programirane celične smrti. Oksidativni stres in apoptoza sta tesno povezana s patogenezo moške neplodnosti. Nivoji reaktivnih oksidativnih molekul (angl. Reactive ozidative species, ROS) so pri moških z idiopatskimi oblikami neplodnosti povišani, motena apoptoza pa bi lahko zavirala maturacijo germinalnih celic v spermije (Agarwal in Said, 2004). Povišano izražanje genov poveznih z odzivom na organske substance potrjuje vpliv dejavnikov okolja na moško neplodnost (Bonde in Giwercman, 1995). Normalna biosinteza steroidnih hormonov je nujna za spermatogenezo in študije so že pokazale na interakcije okoljskih toksinov na steroidne receptorje v testisu (Saradha in Mathur, 2006; Danzo, 1997, Waring in Harris, 2005).

Glede na heterogeno tkivno sestavo testisa in ključno vlogo Sertolijevih in Leydigovih celic pri vseh fazah spermatogeneze, smo preiskali transkriptomske profile testsa ne le na nivoju celotnega vzorca tkiva, temveč tudi na nivoju posameznih celičnih vrst (de Kretser in sod., 1998). Kljub temu da smo na celotnem vzorcu tesisa prikazali obsežne spremembe v izražanju genov, celično specifične analize niso pokazale statistično pomembnih razlik v izražanju genov v populaciji Leydigovih in Sertolijevih celic, pri posameznikih z moteno spermatogenezo v primerjavi s tistimi z normalno spermatogenezo.

MDS analiza (analiza z redukcijo dimenzij, angl. multidimensional scaling) je pokazala na jasno ločitev vzorcev s hudo motnjo spermatogeneze in tistih z normalno spermatogenezo. Kljub temu nismo ugotovili razlik med vzorci pri opazovanju celično specifičnih profilov

Sertolijevih in Leydigovih celic. Glede na jasnost razlik opaženih v celotnem tkivu gre pri tem najverjetneje za prevlado razlik izražanja genov v germinalnih celicah, druga možnost pa je, da razlike v izražanju genov v Sertolijevih in Leydigovih celicah niso dovolj izrazite da bi jih z našim eksperimentalnim pristopom lahko zaznali. Vzrok za to bi lahko izviral iz histološke heterogenosti med različnimi področji testisa pri moških s hudimi motnjami v spermatogenezi. Razporeditev in delež posamične celične vrste je lahko različna v različnih področjih testisa, zato je možno, da izmerjeni deleži za posamezno celično populacijo niso bili dovolj reprezentativni za celotni testis.

S transkriptomsko študijo smo odkrili številne gene povezane z motnjami v spermatogenezi, s čimer smo prispevali k razumevanju sprememb v izražanju genov. Pokazali smo pomen številnih novih genetskih mehanizmov, ki bi bile pomembne tarče za bodoče študije pri moški neplodnosti.

Naši rezultati tako dajejo nove dokaze za povezavo genov cirkadianega sistema z moško neplodnostjo in kažejo na pomembno vlogo cirkadianih ritmov v reprodukciji človeka. Rezultati transkriptomske študije so potrdili pomen vnetja pri patogenezi moške neplodnosti in izpostavili pomen vpliva oksidativnih procesov na plodnost, ter prispevali k razumevanju sprememb v genski ekspresiji pri patogenezi moške neplodnosti.

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ACKNOWLEDGMENTS

I would like to thank to my supervisor, Prof. Dr. Borut Peterlin, for providing me with the opportunity to work on this thesis in his laboratory and the resources necessary to conduct my research. I am extremely grateful because he has been generous with his time and attention during the course of my study, which makes him a caring mentor. He also helped me grow much as a researcher and encouraged me all the time.

I would like to thank to the members of my thesis committee, Prof. Dr. Peter Dovč and Prof. Dr. Radovan Komel, who willingly sacrificed their valuable time to dedicate to my work. I would like to thak them for their efforts and assistance.

I would like to express my appreciation to the members of the Clinical Institute of Medical Genetics, University medical Centre Ljubljana. Their help with many lab tasks has made it possible for me to carry out my research.

I would like to thank dr. Branko Zorn for helping me collect the patients for my research, and prof. dr. Danijel Petrovic for his help with histological and morphometrical analysis.

I would like to thank Mrs. Visam Bajt for revising the English text.

I would also like to express gratitude for financial support of my research work for this dissertation from the Ministry of Higher Education, Science and Technology (Operation Part Financed By The European Union, European Social Fund).

The thesis would not have come to a successful completion, without the help I received from my dear colleague Ales Maver. I would like to thank him for support I got in a very special way. I gained a lot from him and would like to thank him for the sleepless nights we were working together before deadlines and for all the fun we have had.

My deepest and most sincere gratitude goes to my family, to whom I owe a lot, to my father Zuvdija, brothers Danijel and Damir, and specially to my mother Jadranka, who encouraged and helped me at every stage of my personal and academic life, and longed to see this achievement come true.