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Correlation between Histology and Gene Expression in Cryptorchidism

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Medicine and Health Sciences,
Medicine (M 001)

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Academic supervisor:
Assoc. Prof. Dr. Gilvydas Verkauskas (Vilnius University, Medicine and Health Sciences, Medicine – M 001).

Dissertation Defence Panel:
Chairman – **Prof. Dr. Kęstutis Strupas** (Vilnius University, Medicine and Health Sciences, Medicine – M 001);

Members:
Assoc. Prof. Dr. Aušra Matulevičienė (Vilnius University, Medicine and Health Sciences, Medicine – M 001);
Prof. Dr. Eugene Minevich (University of Cincinnati, Medicine and Health Sciences, Medicine – M 001);
Prof. Dr. Tomas Poškus (Vilnius University, Medicine and Health Sciences, Medicine – M 001);
Prof. Dr. Birutė Žilaitienė (Lithuanian University of Health Sciences, Medicine and Health Sciences, Medicine – M 001).

The dissertation shall be defended at a public meeting of the Dissertation Defence Panel at 2 p.m. on April 22, 2021 in the Grand Auditorium (VAA101) at the Vilnius University Hospital Santaros Klinikos.
Address: 7 Santariškių Str., 08406 Vilnius, Lithuania.

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Mokslinis vadovas

Doc. dr. Gilvydas Verkauskas (Vilniaus universitetas, medicinos ir sveikatos mokslai, medicina – M 001).

Gynimo taryba:

Pirmininkas – **prof. habil. dr. Kęstutis Strupas** (Vilniaus universitetas, medicinos ir sveikatos mokslai, medicina – M 001);

Nariai:

Doc. dr. Aušra Matulevičienė (Vilniaus universitetas, medicinos ir sveikatos mokslai, medicina – M 001);

Prof. dr. Eugene Minevich (Sinsinačio universitetas, medicinos ir sveikatos mokslai, medicina – M 001);

Prof. dr. Tomas Poškus (Vilniaus universitetas, Medicinos ir sveikatos mokslai, Medicina – M 001);

Prof. dr. Birutė Žilaitienė (Lietuvos sveikatos mokslų universitetas, medicinos ir sveikatos mokslai, medicina – M 001).

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ABBREVIATIONS

Ad/T	– Ad spermatogonia count per tubule
bp	– base pair – unit of two nucleobases
FC	– fold-change
FDR	– false discovery rate
GnRH	– gonadotropin-releasing hormone
GnRHa	– gonadotropin-releasing hormone analogue
HCA	– hierarchical cluster analysis
HIR	– hight infertility risk
HPG axis	– hypothalamic-pituitary-gonadal axis
LH	– liuteinizing hormone
LHRHa	– liuteinizing hormone-releasing hormone analogue
LIR	– low infertility risk
log_2FC	– binary logarithm of fold-change
MAD	– median absolute deviation
Md	– median
RNA	– ribonucleic acid
AS-lncRNA	– antisense long non-coding RNA
lincRNA	– long intergenic non-coding RNA
lncRNA	– long non-coding RNA
ncRNA	– non-coding RNA
PCA	– principal component analysis
RPKM	– reads per kilo base per million mapped reads
S/T	– spermatogonia count per tubule
TFI	– tubular fertility index
VUH SK	– Vilnius University Hospital Santaros klinikos

1. INTRODUCTION

1.1. Research problem

Undescended testes is one of the most common urogenital condition in newborn boys. In Lithuania the overall incidence of cryptorchidism is 5.7 % (Preikša *et al.*, 2005). In preterm boys this condition is diagnosed more often, up to 18.1 % (Preikša *et al.*, 2005). The incidence of cryptorchidism at age of one year decreases to 1.4 % because of spontaneus testicular descent that occurs in some cases (Preikša *et al.*, 2005). This statement is tightly connected to the problem of correct diagnosis in small boys. Worldwide, the incidence of undescended testes is 2–9 %, and in preterm boys reaches 45.3 % (Cortes *et al.*, 2008; Sijstermans *et al.*, 2008; Bergbrant *et al.*, 2018). Thirty percent of the cases are bilateral (Thorup *et al.*, 2010). The etiology of cryptorchidism is multifactoral – the development of undescended testes is influenced by anatomical, hormonal, genetic and environmental factors. Cryptorchidism may manifest as an isolated anomaly (isolated cryptorchidism) or be the part of other diseases, syndromes or conditions (syndromic cryptorchidism), e.g. Prader-Willi, Kallmann syndromes or disorders of sex development (Urh *et al.*, 2018; Rodprasert *et al.*, 2020). The etiology of undescended testes is mostly unknown. Confirmed risk factors of cryptorchidism are: low birth weight, maternal smoking and gestational diabetes, and obesity before pregnancy (Thonneau, Gandia and Mieusset, 2003; Preikša *et al.*, 2005; Barthold, Reinhardt and Thorup, 2016; Kjersgaard *et al.*, 2018). Hypogonadotropic hypogonadism as a cause of cryptorchidism and infertility has an important history of investigation (Job *et al.*, 1987; Hamza *et al.*, 2001; Hadziselimovic and Hoecht, 2008). In 70 % of the cases undescended testes display the signs of hypogonadotropic hypogonadism: lack of germ cells, impairment of gonocyte transition to Ad spermatgonia, Leydig cell atrophy (Dale S Huff *et al.*, 2001; Hadziselimovic and Hoecht,

2008). Cryptorchidism is associated with infertility and a 5 to 10 times greater risk of developing testicular tumors (Gracia *et al.*, 2000; Pettersson *et al.*, 2007; Walsh *et al.*, 2007; Trsinar and Muravec, 2009; van Brakel *et al.*, 2013). Moreover, it is one of the main etiologic causes of non-obstructive azoospermia in men (Fedder *et al.*, 2004). Up to date, orchidopexy during the first year of life is the recommended treatment of undescended testes (Lee, 2005; Ritzén *et al.*, 2007; Kolon *et al.*, 2014; Radmayr *et al.*, 2016). However, surgical treatment itself does not prevent formerly cryptorchid men from developing non-obstructive infertility (Hadziselimovic and Herzog, 2001; Hadziselimovic, 2006; Trsinar and Muravec, 2009; van Brakel *et al.*, 2013; Adomaitis *et al.*, 2016). Decreased fertility potential is associated with impaired endocrine equilibrium because of underexpressed genes that control the hypothalamic-pituitary-gonadal (HPG) axis and are involved in germ cell development (Ferlin *et al.*, 2007; Hadziselimovic *et al.*, 2009, 2010, 2015, 2016, 2018; F. Hadziselimovic *et al.*, 2011; Docampo and Hadziselimovic, 2015; Gegenschatz-Schmid *et al.*, 2017). In infants, during the period of 30–90 days postpartum, the HPG axis is being activated. This results in a short blood surge of gonadotropins and testosterone (Forest *et al.*, 1974; Winter *et al.*, 1976). The period is called mini-puberty. For the first time it was described in 1973 by Forest et al. During mini-puberty, gonocytes transform to Ad spermatogonia – the stem cells for spermatogenesis. This transformation is testosterone-dependent (Hadziselimovic *et al.*, 2005). In case of cryptorchidism, during mini-puberty, the increase of testosterone concentration in the blood is blunted (Gendrel *et al.*, 1977; Baker, Morley and Lucas, 1988; Hamza *et al.*, 2001; Raivio *et al.*, 2003). This leads to an inadequate transformation of gonocytes to Ad spermatogonia, and as a result – to infertility (Hadziselimovic *et al.*, 2005; Zivkovic, Bica and Hadziselimovic, 2007). A long-term prospective study by Hadziselimovic and Hoecht underscored the importance of normal mini-puberty and presence of Ad

spermatogonia in establishing fertility following successful surgery (Hadziselimovic and Hoecht, 2008).

1.2. Relevance of the study

A literature review of published studies demonstrates the importance of mini-puberty and Ad spermatogonia for intact fertility as well as the advantage of adjuvant hormonal treatment of cryptorchidism in restoring fertility potential (Bica and Hadziselimovic, 1992; Hadziselimovic, Herzog and Barthold, 1997). However, until recently, very few prospectively designed studies investigated these processes.

Genome-wide association studies were performed in order to thoroughly determine the etiology of cryptorchidism-induced infertility (Chalmel *et al.*, 2012; Jan *et al.*, 2017). Prof. F. Hadziselimovic (Liestal, Switzerland), together with a team of researchers from the Cryptorchidism Research Institute, was the initiator of the cryptorchidism genetic research project in Lithuania, in collaboration with the Research Council of Lithuania (European Social Fund) (Hadziselimovic *et al.*, 2018, 2019, 2009, 2010, 2015, 2016, 2017; F Hadziselimovic *et al.*, 2011; F. Hadziselimovic *et al.*, 2011; Docampo and Hadziselimovic, 2015; Gegenschatz-Schmid *et al.*, 2017, 2019). A comparison of boys with a high infertility risk (HIR) and low infertility risk (LIR) determined histologically elucidated gene expression differences between both groups that point out the importance of intact HPG axis for normal spermatogenesis (Hadziselimovic *et al.*, 2009; F. Hadziselimovic *et al.*, 2011). The HIR group showed a decreased expression of *PROK2*, *CHD7*, *FGFR1* and *SPRY4*, resulting in insufficient liuteinizing hormone (LH) secretion and, as a consequnce, – impaired mini-puberty and development of infertility (Hadziselimovic *et al.*, 2016). As mentioned above, it is determined that adjuvant hormonal treatment helps restoring the fertility potential. However, little was known about molecular mechanisms

implicated in the process. Also, there were no data regarding how hormonal treatment affects gene expression in cryptorchid testes. Therefore, joined studies were continued in collaboration with the Cryptorchidism Research Institute (Liestal, Switzerland). Only a small part of these studies is a background of the thesis (Vincel *et al.*, 2018; Hadziselimovic *et al.*, 2019).

1.3. Aim of the study

To determine the histological response to hormonal treatment in boys with cryptorchidism and to summarize relevant gene expression changes.

1.4. Objectives of the study

1. To investigate the effect of adjuvant hormonal treatment with gonadotropin-releasing hormone analogue (GnRHa) on the appearance of Ad spermatotogia.
2. To compare gene expression between different infertility risk groups selected by histology.
3. To summarize changes in the expression of genes involved in HPG axis hormonal control and fertility after adjuvant hormonal treatment.
4. To summarize the expression of non-coding RNAs (ncRNAs) that may be potentially involved in the regulation of spermatogenesis, comparing different infertility risk groups and their response to GnRHa treatment.

1.5. Novelty and practical significance of the study

The benefits and terms of use of adjuvant hormonal therapy for cryptorchidism are still debated. Despite published studies presenting positive effects of adjuvant hormonal treatment in establishing fertility potential (Bica and Hadziselimovic, 1992; Hadziselimovic,

Herzog and Barthold, 1997; D S Huff *et al.*, 2001; Schwentner *et al.*, 2005; Zivkovic, Bica and Hadziselimovic, 2007; Hadziselimovic, 2008; Jallouli *et al.*, 2009; Thorup *et al.*, 2018), the Nordic consensus in 2007 did not include hormonal therapy in their recommendations regarding cryptorchidism treatment (Ritzén *et al.*, 2007). The European Association of Urology, together with the European Society for Paediatric Urology, recommends in its guidelines adjuvant hormonal management in cases of bilateral undescended testes (Kolon *et al.*, 2014), (https://uroweb.org/guideline/paediatric-urology/#3_2). In 2010, Biers and Malone analyzed the level of evidence for improved fertility indices following hormonal treatment in published studies and concluded that adjuvant therapy with gonadotropin-releasing hormone (GnRH) is safe and effective, especially for bilateral cases of cryptorchidism (Biers and Malone, 2010). Our prospective randomized study confirmed the above conclusions. This is important for supporting the concept that adjuvant hormonal therapy should be included in the international guidelines of undescended testes treatment and the consensus to decrease infertility rates in previously cryptorchid men. After the start of the project in collaboration with Prof. F. Hadziselimovic, the Cryptorchidism Research Institute (Liestal, Switzerland), the National Center of Pathology (Vilnius, Lithuania), and the employment of described technique of testicular biopsy processing in 2013, paediatric urologists at the Vilnius University Hospital Santaros Klinikos (VUH SK) Children's Surgery Department follow the protocol of cryptorchidism management: parents consent with possibility to assess the infertility risk of their cryptorchid child based on Ad spermatogonia in testicular biopsy obtained during orchidopexy and, when HIR is diagnosed, the adjuvant GnRHa treatment is adviced. Further research is advocated to employ changes in guidelines for surgical and hormonal treatment.

One of the goals in the field of reproductional biology is to determine molecular mechanisms driving the development of cryptorchidism and to develop an effective treatment of its long-term

consequences. Based on the hypothesis of mini-puberty in early life, during the specific timeframe of hormonal sensitivity, the exposition to sex hormones induces sex / gender-specific developmental processes. It is important that initial transcriptional changes persist to maintain the molecular characteristics of differentiated cells. This study was based on already accomplished genetic studies that investigated molecular mechanisms responsible for development of infertility in cryptorchidism (Hadziselimovic *et al.*, 2009, 2016; F. Hadziselimovic *et al.*, 2011; Docampo and Hadziselimovic, 2015). The novelty and the advantage of collaboration between researchers in different countries allowed to not only perform a prospective histological evaluation but to get insight into molecular mechanisms of pathological findings. Moreover, RNA sequencing revealed important differential expression of long non-coding RNAs (lncRNAs). These lncRNAs may participate in establishing spermatogenesis and fertility potential. Obtained results provide information for further functional analysis of lncRNAs focusing on cryptorchism-induced infertility development (Hadziselimovic *et al.*, 2019).

1.6. Statements to be defended

1. Adjuvant hormonal treatment with GnRHa has a positive effect on restoring fertility potential by inducing gonocyte transition to Ad spermatogonia.
2. Gene expression in undescended testes correlates with histological findings: it significantly differs between testes of high and low infertility risk groups.
3. Adjuvant hormonal treatment with GnRHa improves the expression of genes involved in hormonal control of HPG axis and fertility.
4. Non-coding RNAs play an important role in the regulation of spermatogenesis.

5. GnRHa has a possitive effect on the expression of non-coding RNAs in undescended testes.

2. METHODOLOGY

The study was performed between 2016 and 2020 in VUH SK Children's Surgery Department. Vilnius Regional Bioethics Committee issued the permission (No. 158200-16-883-390 and No. 158200-16-883-PP1-112) to conduct the study. All molecular studies were performed by the Cryptorchidism Research Institute (Liestal, Switzerland) in collaboration with Biozentrum, The Center for Molecular Life Sciences, University of Basel (Basel, Switzerland) and Friedrich Miescher Institute for Biomedical Research (Basel, Switzerland). Consent forms were obtained from both parents or legal guardians.

The study consisted of 5 phases:

1. Patient selection and data collection.
2. Determination of GnRHa effects on gonocyte transition to Ad spermatogonia by evaluating testicular biopsies.
3. Determination of changes in gene expression that are involved in hormonal HPG axis control and fertility, comparing different infertility risk groups and therapy regimens (surgical treatment alone vs. surgical treatment with adjuvant hormonal treatment).
4. Determination of ncRNAs expression and its changes in undescended testes depending on therapy regimen (surgical treatment alone vs. surgical treatment with adjuvant hormonal treatment).
5. Data analysis and interpretation of the results.

Analyzed data and testicular biopsy samples were obtained from cryptorchid patients treated in VUH SK Children's Surgery Department in 2012–2016 who had participated in a prospective randomized study (Vilnius Regional Bioethics Committee approval No. 158200-580-PPI-17).

Histological analysis was performed at the National Center of Pathology. The images were analyzed and described by pathologist

Darius Dasevičius (DD) in collaboration with Prof. Faruk Hadziselimovic (FH) (Liestal, Switzerland).

Molecular studies were performed at Biozentrum, The Center for Molecular Life Sciences, University of Basel by Philippe Demougin. Michael B. Stadler, Friedrich Miescher Institute for Biomedical Research, performed bioinformatic research and statistical analysis of molecular data.

2.1. Characteristics of the study population

2.1.1 Inclusion criteria

- Age: ≤ 6 years;
- No symptoms of acute or chronic disease;
- No syndroms or associated anomalies;
- No confirmed diagnosis of hypogonadotropic hypogonadism;
- No confirmed systemic disease (e.g. hemochromatosis, sarcoidosis, histiocytosis X);
- No confirmed injuries of pituitary gland (tumors, granuloma, abscess);
- No history of hormonal treatment;
- No diagnosis of mobile testes.

2.1.2 Exclusion criteria

- Age: > 6 years;
- Present symptoms of acute or chronic disease;
- Diagnosed syndromes, associated anomalies, hypogonadotropic hypogonadism;
- Presence of systemic disease;
- Pituitary gland injuries;
- Positive history of hormonal treatment;
- Confirmed mobile testes.

2.2. Investigation process

Patient selection: patients referred to the VUH SK Consultative Outpatient Department for paediatric surgeon or urologist consultation and diagnosed with unilateral or bilateral cryptorchidism, meeting the inclusion criteria and appointed for surgical treatment in VUH SK Children's Surgery Department.

Consent: both parents or legal guardians of patients to be enrolled in the study were acquainted with the research and signed the Consent form.

Clinical evaluation on the day of surgery:

1. Anamnesis:
   - when the absence of testes in the scrotum was noticed;
   - are there any diagnosed endocrinological conditions or syndroms;
   - history of any hormonal treatment applied previously in life.
2. General physical examination: weight, height, appearance and development of scrotum and external genitalia.
3. Special physical examination:
   - Patients were examined in the supine position palpating with warm hands.
   - Determination of the position of testes: external inguinal ring, inguinal canal, abdominal cavity (non-palpable).
   - Exclusion of retractile testes. Retractile testes can be brought to the bottom of the scrotum and, when the child is peaceful, they stay there. Undescended testes can not be brought to the bottom of the scrotum and, when brought to the middle and released, they immediately return to the groin.

- Secondary evaluation of the testicular position before the surgery under the general anesthesia (important for selecting surgery method – open surgery or laparoscopy – when testes are non-palpable during physical examination).

Workflow of the study

Forty-seven patients were enrolled in the study and two research groups were formed (Figure 1).

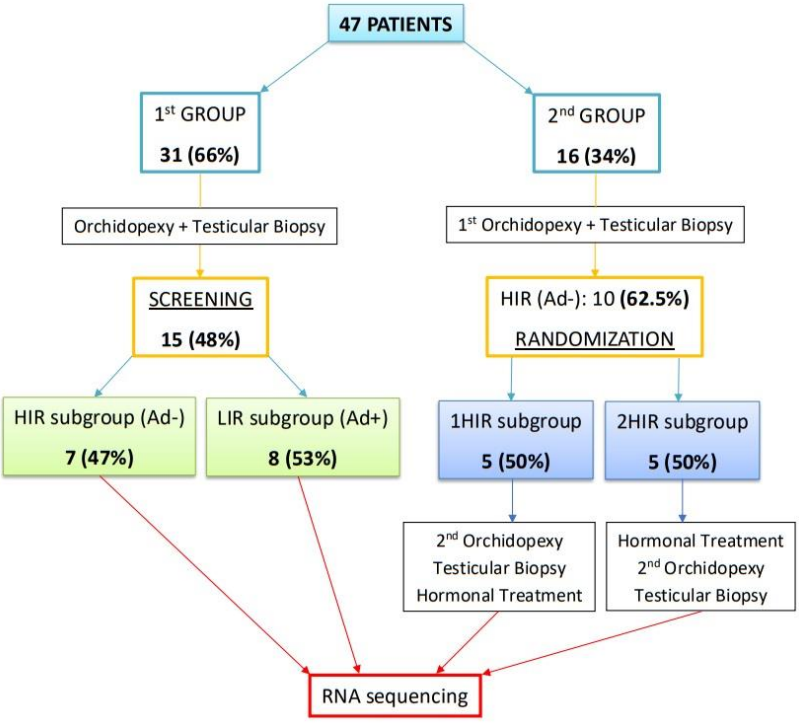


Figure 1. *Workflow of the study.*

The first group included 31 (66 %) patients with unilateral or bilateral cryptorchidism, of which 15 (48 %) successfully finished

the study. During orchidopexy, a testicular biopsy for histological and molecular analyses was obtained. Histological analysis and description were performed by two pathologists (DD – primary evaluation, FH – secondary evaluation and double-check). Based on the histological evaluation (presence or abscence of Ad spermatogonia in testicular biopsy), the first group was divided into two subgroups: HIR (7 (47 %)) – high infertility risk (no Ad spermatogonia) and LIR (8 (53 %)) – low infertility risk (Ad spermatogonia present). Patients were aged 7 to 55 months (median 15 months). All testes were located in the groin region.

The second group was formed from 16 (34 %) patients with isolated bilateral cryptorchidism. During the first orchidopexy, a testicular biopsy was performed for histological and molecular analyses. Two pathologists (DD – primary evaluation, FH – double-check) conducted the histological evaluation. Based on the results, patients were allocated to the HIR (no Ad spermatogonia) or LIR (Ad spermatogonia present) subgroup. Patients who belonged to the LIR subgroup (five boys) were excluded from further research. Parents of one boy assigned to the HIR subgroup refused to continue the study for personal reasons. Ten (62.5 %) remaining patients were randomized and successfully finished the study. They were divided into two subgroups with 5 patients in each arm regarding the adjuvant hormonal treatment time: 1HIR – second orchidopexy −> hormonal treatment, 2HIR – hormonal treatment −> second orchidopexy. Hormonal therapy with liuteinizing hormone-releasing hormone analogue (LHRHa) nasal spray (10 μg) delivered every second day in the evening for 6 months was applied. The dose was selected based on the pharmacokinetic study performed by Sandow et al. (Sandow *et al.*, 1984). Ipsilateral testicular biopsy was performed during the second orchidopexy. A histological evaluation was conducted in the same manner as for the first biopsy. Patients from the 1HIR subgroup who lacked Ad spermatogonia in the second testicular biopsy received identical hormonal treatment as patients from the 2HIR subgroup after the first surgery. During the first

surgery, patients were 7–55 months old, and during the second surgery – 10–62 months. A localization of testes is provided in Table 1.

Table 1. *Position of testes during orchidopexies in patients from second research group.*

POSITION OF CRYPTORCHID TESTES	
1st surgery	2nd surgery
1HIR SUBGROUP	
Peritoneal cavity	Peritoneal cavity
Peritoneal cavity	Inguinal canal
Inguinal canal	Inguinal canal
Peritoneal cavity	Peritoneal cavity
Inguinal canal, upper 1/3	Inguinal canal
2HIR SUBGROUP	
Inguinal canal, at external ring	Ectopic, perineum
Inguinal canal	Inguinal canal
Inguinal canal, upper 1/3	Inguinal canal, upper 1/3
Inguinal canal, upper 1/3	Inguinal canal
Inguinal canal, at external ring	Inguinal canal

RNA sequencing was performed from samples obtained from both research groups. An analysis of genes involved in the hormonal control of the HPG axis and fertility and the ncRNAs expression was executed.

An investigation of the GnRHa effect on restoring fertility potential was carried out in the randomized second research group. 1HIR and 2HIR subgroups were compared. Subgroups were age-equivalent during both surgeries with age medians: 1st orchidopexy: 20 and 22 months ($p = 0.3173$), 2nd orchidopexy: 27 and 30 months ($p = 0.1797$).

Gene expression analysis in undescended testes was performed from 15 testicular biopsies obtained from the first research group. The samples from HIR and LIR subgroups were compared. ANOVA was performed to evaluate the possible impact of age on patient subgroups (HIR, LIR) and gene expression. Age did not significantly associate with research subgroups (one-way ANOVA, $p = 0.349$) and was excluded as a factor in data analysis (Hadziselimovic *et al.*, 2016).

To investigate the effect of GnRHa on gene expression, 29 samples obtained from 22 patients were analyzed: 15 patients belonged to the first research group (15 samples) and 7 patients – to the second research group (14 samples) (Figure 1). First, the differences in gene expression between HIR (7 samples) and LIR (8 samples) subgroups in the first research group were determined. Then, the impact of adjuvant hormonal treatment was analyzed comparing 1HIR (3 patients, 6 samples) and 2HIR (4 patients, 8 samples) subgroups from the second research group (Hadziselimovic *et al.*, 2017).

The expression pattern of ncRNAs and changes in their expression, induced following the GnRHa treatment, were analyzed using samples obtained from the second research group (Hadziselimovic *et al.*, 2019).

2.3. Testicular biopsy: sample collection and histological evaluation

Testicular biopsy execution and histological analysis were performed according to a protocol mastered during V. Bilius's doctoral studies (Bilius, 2015).

During orchidopexy, a rice grain-sized sample of testicular tissue was obtained at the most lateral testicular curvature opposite the epididymis. Specimen was divided into 2 parts: one half – for histological evaluation, other half – for molecular analysis

(immediately immersed in *RNAlater©* (*ThermoFisher Scientific*, USA) and stored at -25°C temperature until further processing).

Testicular tissue specimens designed for histological evaluation during surgery were immersed in 2 % glutaraldehyde solution and transfered to the National Center of Pathology. They were fixed ≥ 12 h and then embedded in Epon resin. Later, semi-thin sections, 1 μm thick, were cut on a *Leica EM UC6* (*Leica microsystems*, Germany) ultramicrotome, mounted on standart glass slides and stained with 1 % toluidine blue. A preliminary evaluation was conducted under the *Olympus BX40F4* light microscope (*Olympus Optical Co LTD*, Japan) at a magnification of 400-600x. A detailed specimen analysis was performed examining digital images. At least 50 tubular cross sections per biopsy were evaluated. Special attention was payed to the identification of Ad spermatogonia (Figure 2), the distinctive features of which (typical halo in the nucleus – rarefication zone, darker appearance of cytoplasm) were first published by Seguchi and Hadziselimovic in 1974 (Seguchi and Hadziselimović, 1974).

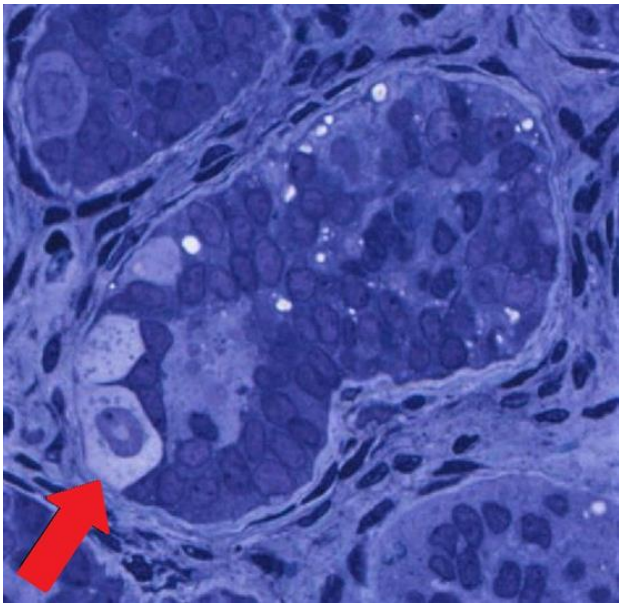


Figure 2. *Testicular biopsy stained with toluidine blue at 400x magnification: tubular cross section with Ad spermatogonia (red arrow). Author: pathologist D. Dasevičius.*

Parameters analyzed in testicular biopsies:

1. number of tubular cross sections per biopsy;
2. number of tubules with spermatogonia per biopsy;
3. number of spermatogonia per biopsy;
4. number of Ad spermatogonia;
5. tubular fertility index (TFI) – ratio of number of tubules with spermatogonia to number of tubular cross sections per biopsy;
6. number of Ad spermatogonia per tubule (Ad/T) – ratio of number of Ad spermatogonia to number of tubular cross sections per biopsy;
7. spermatogonia count per tubule (S/T) – ratio of number of spermatogonia to number of tubular cross sections in biopsy.

2.4. RNA extraction (Hadziselimovic *et al.*, 2016)

2.4.1 RNA isolation and purification

Total RNA was isolated from testicular biopsies immersed in *RNAlater©*. Biologic matter was homogenized using the *TissueLyser II* (*Qiagen*, Germany) device: tissues were mixed with 500 µL of *TRI-Reagent* (*Ambion*, USA) solution and homogenized for 2 min. at 30 Hz frequency. Next, 500 µl of absolute ethanol was added to each sample and mixed. The obtained mixtures were loaded onto *Zymo-Spin IIC* columns (*Zymo Research*, USA). RNA was further purified using a *Direct-Zol* kit (*Zymo Research*, USA) with deoxyribonuclease I to remove any contaminating genomic DNA. The concentration of the eluted RNA was determined using a *NanoDrop 2000* (*Thermo Scientific*, USA) spectrophotometer. Quality was controlled with bioanalyzer *2100 BioAnalyzer* using *RNA 6000 Pico* chips (everything from *Agilent*, USA).

2.4.2 RNA library preparation

Library preparation was accomplished from 300 ng total RNA using *TruSeq Stranded Total RNA* kit with *Ribo-Zero Gold* (*Illumina*, USA). Ribosomal RNA was removed to facilitate the sequencing of coding and non-coding RNAs as well as the ligation of specific indexes. Quality control was performed on *Fragment Analyzer* using *Standard Sensitivity NGS Fragment Analysis* kit (everything from *Advanced Analytical*, USA). Libraries were of excellent quality (average concentration: 128 nmol/L; average size: 367 bp). Prepared RNA libraries were pooled to 2 equally large series of samples with equivalent molarity. Each pool was quantificated and quality-controlled on *Fragment Analyzer*.

2.4.3 RNA sequencing

Library pools (concentration: 1.3 and 1.4 pM) were used for clustering on the *NextSeq 500* (*Illumina*, USA) instrument. The sequencing of each pool was performed on two flow-cells to yield more reads. This increases the ability to detect poorly expressed transcripts and ncRNAs. Samples were sequenced as singlereads of 81 bases using the *NextSeq 500/550 High Output Kit v2* (75 cycles) (*Illumina*, USA). The pools yielded 91–95 % of pass-filter reads (equivalent to *Illumina Q30* quality score). Primary data analyses were performed with *Illumina RTA (Real Time Analysis) v.2.4.6* and *bcl2fastq v2.17.1.14* software.

2.4.4 RNA sequencing data analysis

Reads were aligned to the *UCSC human genome browser hg19 assembly (GRCh37)* using *STAR v.2.5.0a* RNA-Seq aligner. Around 60 million reads were mapped per sample. On average, 96 % of reads were mapped with less than 10 % of multimappers. A random subset of reads from each sample was also aligned to a collection of alternative references in order to identify any potentially contaminating ribosomal RNA or nucleic acids from other species. Raw data files are available at the *Database of Genotypes and Phenotypes (dbGap)* (phs001275.v1.p1).

2.4.5 RNA expression level quantification

To quantify gene expression, alignments were counted per gene and sample using the *QuasR Bioconductor* package (Gaidatzis *et al.*, 2015) with known genes from the *UCSC knownGene* table (*TxDb.Hsapiens.UCSC.hg19.knownGene Bioconductor package*) and with ncRNAs from the *UCSC lincRNAsTranscripts* table (*TxDb.Hsapiens.UCSC.hg19.lincRNAsTranscripts Bioconductor package, v.3.2.2*). Raw data (mapped read counts per gene) were

normalized for RNA length and for total read number in the lane to RPKM – reads per kilo base per million mapped reads. Data normalization enables the removal of technical biases and a comparison of transcripts.

RPKM = numReads / (geneLength/1000 * totalNumReads/1,000,000)

numReads – number of reads mapped to a gene sequence
geneLength – length of the gene sequence
totalNumReads – total number of mapped reads of a sample (kb)

2.5. Statistical analysis

Statistical analysis was performed with Microsoft Excel and the open-access statistical software R. Data for qualitative variables are presented as the absolute score (n) and the percentage of the analyzed sample (%). Quantitative variables are presented as mean, median (Md) and mean absolute deviation (MAD). Due to small sample size nonparametric statistical methods were used. The *Wilcoxon signed-rank test* was used for dependent samples to compare medians. *Fisher's exact test* was used to compare categorical values. *ANOVA* was used to compare subgroups and differences in gene expression. $p < 0.05$ was considered statistically significant.

2.5.1 Data and differential gene expression analyses (Hadziselimovic *et al.*, 2016, 2017)

Normalized data (RPKM) for all samples were analyzed. *Principal component analysis* (PCA) and *hierarchical cluster analysis* (HCA) were applied to identify different patterns in the dataset and highlight similarities and differences among the samples.

To determine genes differentialy expressed in HIR versus LIR subgroups and before versus after hormonal treatment (1HIR versus 2HIR subgroups), R software with the *edgeR* package (Robinson,

McCarthy and Smyth, 2009) was used with implemented *quasi-likelihood* methods (Lund *et al.*, 2012). A statistical model with two additional categorical factors was designed: 1 – *patient* – determines measurements from the same sample before and after GnRHa treatment and controls for gene expression differences between individuals; 2 – *treatment* – models gene expression changes induced by GnRHa treatment.

Analysis was performed on genes with at least one read per million in at least two samples. Differentially expressed genes – genes with false discovery rate (FDR) < 0.05 and at least 2-fold change (FC) in expression. Changes in gene expression are presented as a binary logarithm of FC ($\log_2$FC) that is applied in order to compare downregulated and upregulated genes.

FDR – method applied when hypothesis is being tested several times in order to rule out random "discoveries" that seem to be significant, i.e., obtaining a false positive result (incorrectly rejected null hypothesis). When applying this method statistical power is usually being increased and there are fewer type I errors. FDR was calculated after the Benjamini and Hochberg approach (Benjamini and Hochberg, 1995; Rouam, 2013) (https://www.statisticshowto.com/false-discovery-rate/).

2.5.2 LncRNA data interpretation (Hadziselimovic *et al.*, 2019)

The expression of long intergenic non-coding RNAs (lincRNAs) and antisense long non-coding RNAs (AS-lncRNAs) in cryptorchid testes with HIR before (1HIR subgroup) versus after (2HIR subgroup) adjuvant hormonal treatment with GnRHa was analyzed. Moreover, lincRNA and AS-lncRNA expression was compared between HIR and LIR subgroups.

LncRNAs, included in further analysis:

- with $\log_2$FC > 1.0;
- with lower expression in HIR subgroup.

LincRNAs and AS-lncRNAs were selected based on RNA-RNA interactions, revealing the ones important and involved in spermatogenesis or fertility, cell division and growth, signaling pathways, or cancer development. Determined AS-lncRNA candidates were prioritized based on a *PubMed* literature search (www.ncbi.nlm.nih.gov/pubmed). RNA annotation was verified using *Ensembl* database (www.ensembl.org, 97 release) (Yates *et al.*, 2020). The LincRNA/mRNA expression was interpreted using *GermOnline* (www.germonline.org, version 4.0) (Lardenois *et al.*, 2010), *Human Protein Atlas* (www.proteinatlas.org, version 18) (Uhlén *et al.*, 2015; Thul *et al.*, 2017), *Genevestigator*® (www.genevestigator.com, version 7.3.1) (Hruz *et al.*, 2008). Experimentally validated RNA-RNA interaction data were retrieved from the *RISE* database (http://rise.life.tsinghua.edu.cn, version 1.0) (Gong *et al.*, 2018).

3. RESULTS

3.1. GnRHa effects on male fertility potential

Adjuvant hormonal treatment with GnRHa given 10 μg intranasally every second evening for a period of 6 months was generally well-tolerated. One patient discontinued the treatment after 1 month due to an apparent burning sensation. Nevertheless, even short-term administration of GnRHa had a positive effect on fertility potential: S/T increased from 0.08 to 0.13 and Ad spermatogonia appeared (Table 2).

S/T did not differ (p = 0.67) between 1HIR and 2HIR subgroups during first surgery. After the second orchidopexy 1HIR subgroup exhibited changes in S/T as follows: significant increase – 1 patient, slight increase – 2 patients, decrease – 1 patient (Figure 3). Contrary results were observed in the 2HIR subgroup: S/T increased in all 5 patients with the median 0.11 before and 0.42 after GnRHa treatment (p = 0.03; Wilcoxon signed-rank test, one-tailed) (Figure 4). Ad spermatogonia appeared only in patients from 2HIR subgroup who received adjuvant hormonal treatment (p = 0.008; Fisher test, 2-tailed) (Table 2).

Table 2. *Histological data of testicular biopsies obtained from second research group.*

Age, months	Ad/T1	Ad/T2	S/T1	S/T2	TFI1	TFI2
1HIR SUBGROUP						
10	0	0	0.18	0.15	0.2428	0.1573
7	0	0	0.32	0.34	0.1346	0.3125
20	0	0	0.11	0.12	0.0833	0.1143
22	0	0	0.33	0.06	0.2367	0.0492
53	0	0	0.15	0.43	0.0413	0.6029

Continued table.

2HIR SUBGROUP						
13	0	0.0085	0.08	0.13	0.0933	0.2479
32	0	0.0109	0.47	0.77	0.6136	0.7717
55	0	0.0056	0.07	0.42	0.0612	0.3966
22	0	0.005	0.11	0.40	0.1419	0.2475
10	0	0.0208	0.13	0.79	0.8947	0.7917

Ad/T1: number of Ad spermatogonia per tubule in first testicular biopsy
Ad/T2: number of Ad spermatogonia per tubule in second testicular biopsy
S/T1: spermatogonia count per tubule in first testicular biopsy
S/T2: spermatogonia count per tubule in second testicular biopsy
TFI1: tubular fertility index in first testicular biopsy
TFI2: tubular fertility index in second testicular biopsy

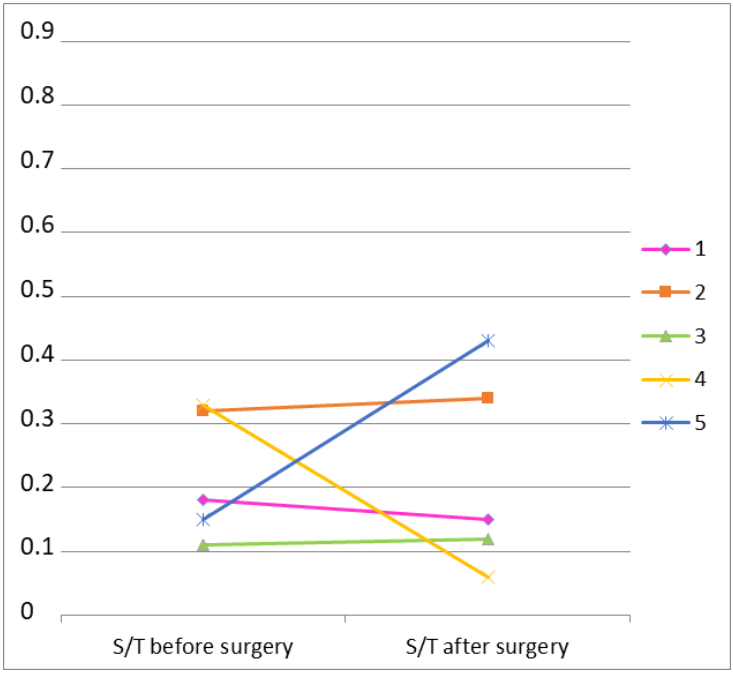


Figure 3. *S/T changes in 1HIR subgroup.* Every colour represents one patient.

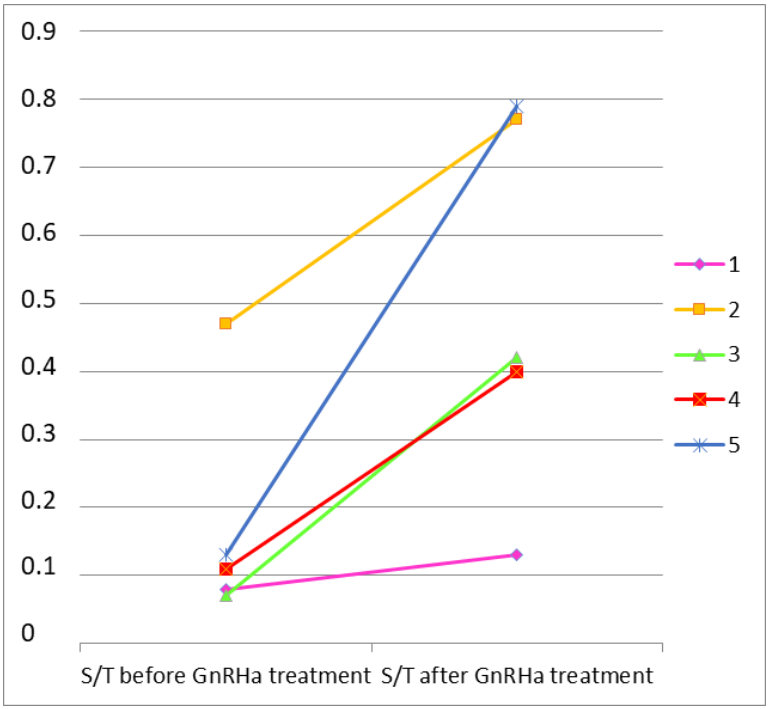
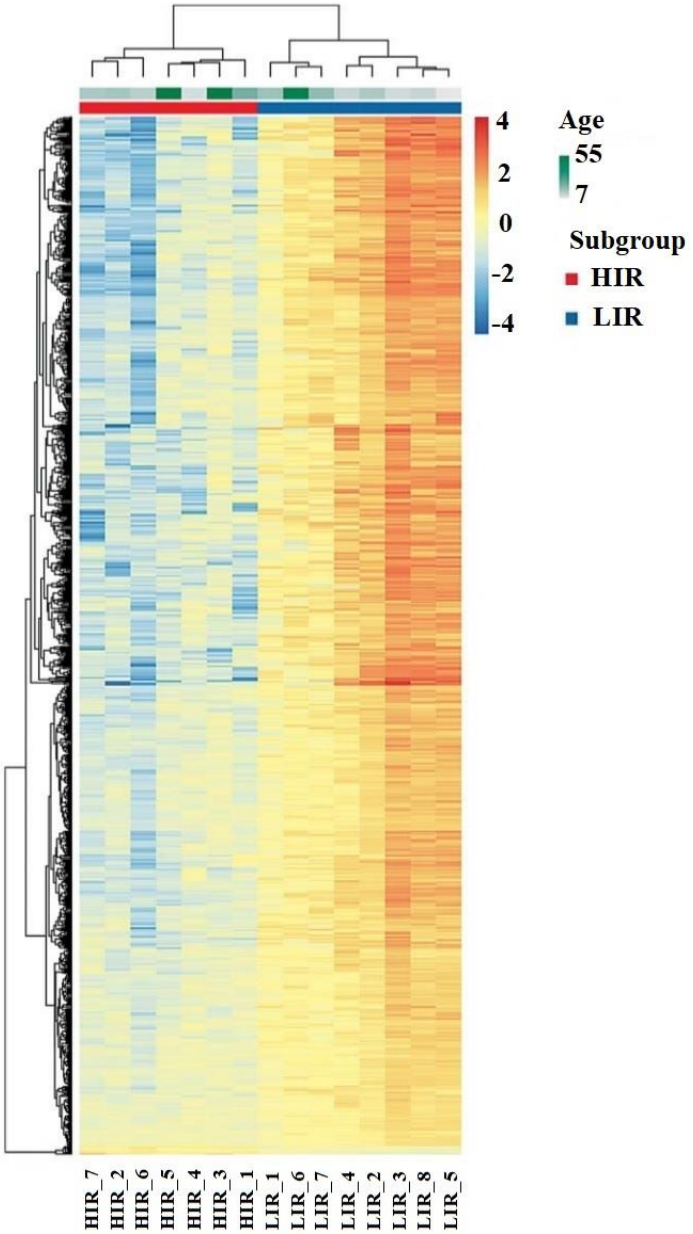


Figure 4. *S/T changes in 2HIR subgroup.* Every colour represents one patient.

3.2. Gene expression in cryptorchid testes (Hadziselimovic *et al.*, 2016)

From the samples obtained from the first research group, 23 737 genes were detected; 2 033 genes had significant difference in expression between HIR and LIR subgroups (FDR < 0.05; FC ≥ 2). 97.7 % (1 986) of the genes had lower expression in the HIR subgroup compared to LIR, and only 2.3 % (47) had a higher expression. The following heatmap represents gene expression patterns of the 1 000 most significant differential genes (Figure 5).



Continued on next page

Figure 5. *Heatmap of gene expression pattern in cryptorchid testes.* The $\log_2$FC of 1 000 most significant differential genes between low (LIR) and high (HIR) infertility risk subgroups is represented. Patient subgroups and age (months) are colour-coded and indicated on the top of the heatmap. The colour scale indicates the intensity of expression: red – high, blue – low. Samples and genes are ordered in groups by hierarchical clustering (dendrograms on the top and left side, respectively) (Hadziselimovic *et al.*, 2016).

3.3. GnRHa effects on gene expression in cryptorchid testes (Hadziselimovic *et al.*, 2017)

From the samples obtained from the 2HIR subgroup, 28 645 genes were determined; 6 469 genes showed difference in expression before and after hormonal treatment. Increased gene expression after GnRHa treatment was detected in 90 % of the cases (FDR < 0.05; FC ≥ 2).

Differential expression analysis of 1 000 most significant differential genes was performed. Samples from 1HIR subgroup were paired before / after surgical treatment (Surgery 1 / Surgery 2) and from 2HIR subgroup – before / after adjuvant hormonal therapy (GnRHa- / GnRHa+). Results are represented as a heatmap with a dendrogrogram (Figure 6). Analysis showed that orchidopexy alone (1HIR subgroup) induces very few significant changes in gene expression (Figure 6, compare lanes: 1 versus 3, 2 versus 4). Whereas the application of adjuvant GnRHa therapy (2HIR subgroup) enhances gene expression in most of the cases (Figure 6, compare lanes: 5 versus 7, 6 versus 8).

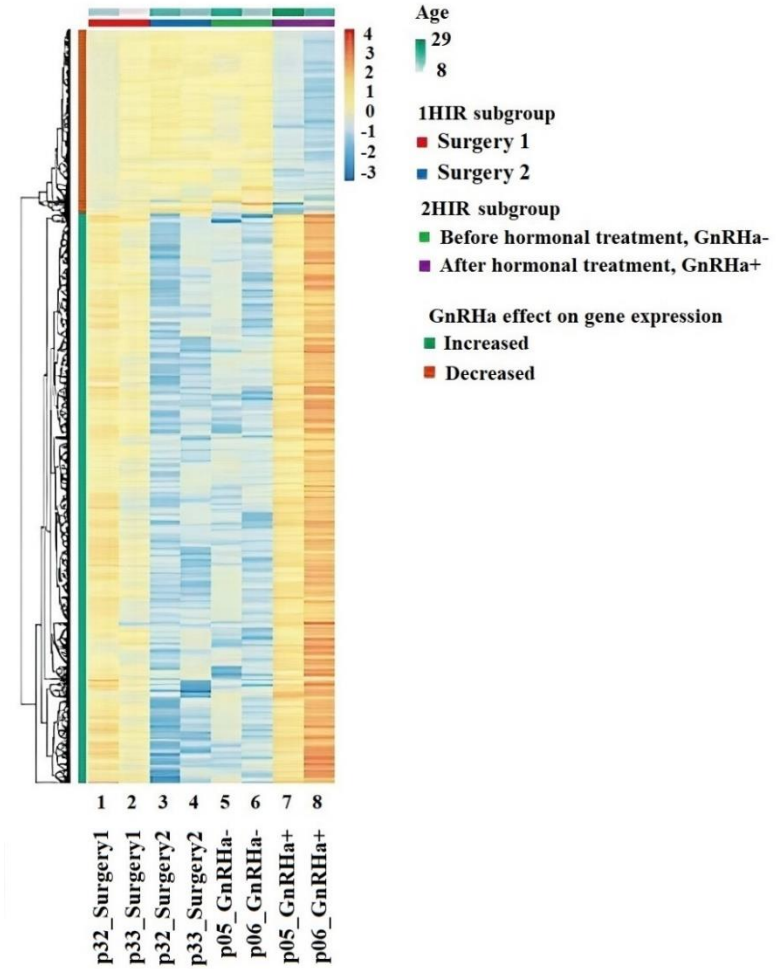


Figure 6. *Heatmap of gene expression pattern in high infertility risk (HIR) cryptorchid testes.* The $\log_2$FC of 1 000 most significant differential genes before / after surgical treatment (Surgery 1 / Surgery 2) and before / after adjuvant hormonal treatment (GnRHa- / GnRHa+) is represented. Patient subgroups and age (months) are colour-coded and indicated on the top of the heatmap. The colour scale indicates the intensity of expression: red – high, blue – low. Genes are ordered in groups by hierarchical clustering (dendrogram on the left side) (Hadziselimovic *et al.*, 2017).

The investigation of GnRHa impact on decreased gene expression in the HIR subgroup revealed a positive effect of adjuvant hormonal treatment – an increase in the expression of formerly downregulated genes was observed. Genes that responded to GnRHa therapy are involved in:

- regulation of gene expression: *DLX3*, *DLX6*, *EGR2*, *FOXG1*, *ISL2*, *NHLH2*, *OTX2*, *PAX7*, *POU3F2*, *POU6F2*, *RGS7*, *SOX1*, *SOX30*;
- chromatin modification: *PRDM9*;
- signal transduction: *NOS1*.

Moreover, the analysis of samples obtained from the first research group revealed that hormonal treatment upregulated genes with no difference in expression between HIR and LIR subgroups. These included:

- DNA/chromatin binding regulators important for development: *DLX1*, *EGR3*, *FGF5*, *LHX4*, *OTX1*, *PAX1*, *PAX2*, *PAX3*, *PAX4*, *PAX6*, *POU1F1*, *POU2AF1*, *POU2F2*, *POU2F3*, *POU4F2*, *PROP1*, *RUNX2*, *SOX2*;
- epigenetic factors involved in histone modification: *PRDM12*, *PRDM16*.

GnRHa upregulated genes important for pituitary development that were downregulated in the HIR subgroup. It is assumed that *FOXG1* is involved in GnRH neuron development (Duggan *et al.*, 2008; Garaffo *et al.*, 2015) in the same manner as *DLX3* – the transcriptional activator of *GnRHR* and *αGSU* (Xie *et al.*, 2013). Both genes were downregulated in the HIR subgroup and upregulated following hormonal treatment. Also, GnRHa treatment stimulated the expression of *ISL2*, together with its paralogue *LHX4* and *OTX2*. *ISL2* is involved in neural stem cell differentiation and in the derivation of lineage-specific markers (Hobert and Westphal, 2000; Li *et al.*, 2007). *LHX4* is a member of LIM-homeobox family that plays an important role in motor neuron development (Liu *et al.*, 2002) and encodes the transcription factor involved in the early

development of the pituitary gland (Cohen *et al.*, 2017). During the development of murine GnRH neurons, upregulation of *OTX2* results in an enhanced production of the homeodomain protein OTX2, which is responsible for increased GnRH promoter activity in GnRH-neuronal cell lines and ensures reproductive competence (Diaczok *et al.*, 2011). Moreover, hormonal treatment upregulated genes with no difference in expression between HIR and LIR subgroups. *SOX2* plays an important role in neural progenitor cell proliferation and maintanance (Graham *et al.*, 2003). *PROP1* is a pituitary-specific transcription factor that plays an important role in pituitary organogenesis and hormone-producing cell differentiation (Nishimura *et al.*, 2016). It was determined that *SOX2* can modulate *PROP1* expression (Nishimura *et al.*, 2016). Moreover, *SOX2* is also linked to proteins PRDM14 and PRDM16. PRDM family proteins control nervous system patterning and the modulation of neuronal progenitor cell proliferation and differentiation (Kinameri *et al.*, 2008). It was determined that GnRHa treatment increased the expression of four *PRDM* genes: *PRDM7*/*9*/*12*/*16*.

The effects of GnRHa on genes involved in testosterone synthesis were diverse (Table 3). Hormonal treatment upregulated two genes – *RGS7* and *AKR1B15* – that were determined to be downregulated in the HIR subgroup. *RGS7* is involved in hormone release and control of endocrine functions (Nini *et al.*, 2012). *AKR1B15* is the new member of the aldo-keto-reductase (AKR) superfamily and encodes protein with potential roles in steroid metabolism, regulation of mitochondrial function, aging (Weber *et al.*, 2015). It was the only underexpressed gene in HIR subgroup from the AKR superfamily members that were analyzed. Most of the analyzed genes involved in testosterone synthesis did not exhibit the significant differences in expression when comparing HIR and LIR subgroups. However, positive response to GnRHa treatment was observed with significant increase in the expression of genes:

- encoding enzymes: *AKR1D1*, *CYP11B1*, *CYP11B2*, *CYP19A1*, *HSD3B1*, *HSD17B2*;

- involved in nonclassical testosterone pathway: *GPRC6A*;
- involved in nonclassical androgen signaling pathway: *FGF23*.

However, some genes were found to be underexpressed after the treatment (*CYP17A1*, *CYP11A1*, *CYPB5B*, *HSD17B1/B4/B11/B12*).

Table 3. *Differential expression of genes, involved in testosterone synthesis, in cryptorchid testes. Modified from* (Hadziselimovic *et al.*, 2017).

Gene ID	**$log_2FC^{HIR/LIR}$**	**$FDR^{HIR/LIR}$**	**$log_2FC^{GnRHa+/-}$**	**$FDR^{GnRHa+/-}$**
AKR1B15	-1.04	0.05	1.63	0.00
AKR1D1	n.s	n.s	1.89	0.00
CYB5B	n.s	n.s	-0.77	0.00
CYP11A1	n.s	n.s	-1.13	0.00
CYP11B1	n.s	n.s	3.44	0.00
CYP11B2	n.s	n.s	2.59	0.00
CYP17A1	n.s	n.s	-1.40	0.00
CYP19A1	n.s	n.s	2.51	0.00
FGF23	n.s.	n.s.	2.69	0.00
GPRC6A	n.s.	n.s.	1.91	0.00
HSD3B1	n.s	n.s	1.83	0.01
HSD17B1	n.s	n.s	-0.93	0.00
HSD17B2	n.s	n.s	2.37	0.01
HSD17B4	n.s	n.s	-0.95	0.00
HSD17B11	n.s	n.s	-0.89	0.00
HSD17B12	n.s	n.s	-0.93	0.00
RGS7	-1.64	0.00	0.75	0.04

Continued table.

Abbreviations: HIR – high infertility risk subgroup samples; LIR – low infertility risk subgroup samples; GnRHa- – 2HIR subgroup samples before hormonal treatment, GnRHa+ – after hormonal treatment; log_2FC – binary logarithm of fold-change; FDR – false discovery rate; n.s. – not significant.

The comparison of differences in gene expression between first and second research groups showed that, in most cases, GnRHa upregulated genes that had lower expression in the HIR subgroup compared to LIR. Data analysis disclosed that adjuvant hormonal treatment, too, stimulated alternative pathways, the entire set of gene families, or their homologues.

Bone morphogenetic proteins (BMPs) belong to the family of transforming growth factor β (TGF-β) and play an important role in many processes during embryonic development and adult homeostasis (Itoh, Watabe and Miyazono, 2014; Katagiri and Watabe, 2016). Based on evidence provided in the literature, it was suggested that BMP signals play an exceptional role in cancer development and progression by modulating both cancer cells and tumor microenvironment. Depending on the context, BMPs can act as both tumor supressors or promoters (Ehata *et al.*, 2013). HIR subgroup exhibited downregulated *BMP6*, *BMP7* and *BMP8A* (Table 4). Their expression after GnRHa treatment remained unaltered. However, *BMP3*, *BMP5* and *BMP10* exhibited 2–4-fold increase in expression following adjuvant hormonal treatment (Table 4).

Similar changes were observed in the chemokine ligand and chemokine receptor families (CXCL, CXCR). Chemokines play a fundamental role in development, homeostasis, immune and inflammatory processes, tissue repair, innate and adaptive immunity (Hughes and Nibbs, 2018). Moreover, chemokines participate in angiogenesis and are expressed in the central nervous system, where they are likely involved in the development of neurodegenerative diseases and inflammatory processes (Cartier *et al.*, 2005; Kiefer and

Siekmann, 2011). A reduced expression of *CXCL9* and *CXCR4* was observed in the HIR subgroup, which the GnRHa treatment failed to enhance (Table 4). However, hormonal treatment increased the expression of other CXCL and CXCR family members: *CXCL3/5/6/8/11/13* and *CXCR1/2/5/6*, which expression did not differ comparing HIR and LIR subgroups (Table 4). Based on the results it can be suggested that *CXCL* and *CXCR* are involved in the differentiation of Ad spermatogonia.

Table 4. *Differential expression of genes encoding bone morphogenetic proteins (BMP), chemokine ligands (CXCL) and chemokine receptors (CXCR). Modified from* (Hadziselimovic *et al.*, 2017)

Gene ID	**$log_2FC^{HIR/LIR}$**	**$FDR^{HIR/LIR}$**	**$log_2FC^{GnRHa+/-}$**	**$FDR^{GnRHa+/-}$**
BMP3	n.s.	n.s	0.92211	0.00394
BMP5	n.s	n.s	2.31201	0.00020
BMP6	-0.76124	0.02666	n.d.	n.d.
BMP7	-1.58963	0.00144	n.d.	n.d.
BMP8A	-0.86631	0.03160	n.d.	n.d.
BMP10	n.s	n.s	2.13774	0.00475
CXCL3	n.s	n.s	1.61585	0.00974
CXCL5	n.s	n.s	1.90972	0.00007
CXCL6	n.s	n.s	2.04525	0.00206
CXCL8	n.s	n.s	1.78386	0.01270
CXCL9	-1.98042	0.00121	n.d.	n.d.
CXCL11	n.s	n.s	1.94361	0.00893
CXCL13	n.s	n.s	3.12194	0.00138
CXCR1	n.s	n.s	2.41284	0,00403

Continued table.

Gene ID	$log_2FC^{HIR/LIR}$	$FDR^{HIR/LIR}$	$log_2FC^{GnRHa+/-}$	$FDR^{GnRHa+/-}$
CXCR2	n.s	n.s	1.73513	0.00137
CXCR4	-0.80011	0.01059	-0.59637	0.02908
CXCR5	n.s	n.s	1.93683	0.02545
CXCR6	n.s	n.s	1.96231	0.00461

Abbreviations: HIR – high infertility risk subgroup samples; LIR – low infertility risk subgroup samples; GnRHa- – 2HIR subgroup samples before hormonal treatment, GnRHa+ – after hormonal treatment; log_2FC – binary logarithm of fold-change; FDR – false discovery rate; n.s. – not significant, n.d. – not determined.

In certain cases, the GnRHa treatment enhanced not only the expression of genes downregulated in the HIR subgroup but also increased the expression of their family members, which did not exhibit any differential expression between HIR and LIR subgroups (Table 5). The analysis of the expression patterns between HIR and LIR subgroups of *DLX* (*distal-less homeobox*) family members showed that *DLX1* expression did not differ, while *DLX3* and *DLX6* were downregulated in the HIR subgroup (respectively: $log_2FC = -2.07726$, $FDR = 0.00661$ and $log_2FC = -2.19564$, $FDR = 0.00030$). The GnRHa treatment enhanced the expression of all three members (respectively: $log_2FC = 1.40060$, $FDR = 0.01565$; $log_2FC = 1.43846$, $FDR = 0.01841$ and $log_2FC = 1.10459$, $FDR = 0.04308$). Such changes in expression were observed as well among members of *EGR*, *OTX*, *PAX*, *RUNX*, *SIX*, *SOX* and POU class families (Table 5).

Table 5. *Genes significantly upregulated following GnRHa treatment. Modified from* (Hadziselimovic *et al.*, 2017)

Gene ID	$log_2FC^{HIR/LIR}$	$FDR^{HIR/LIR}$	$log_2FC^{GnRHa+/-}$	$FDR^{GnRHa+/-}$
DLX1	n.s.	n.s.	1.40060	0.01565
DLX3	-2.07726	0.00661	1.43846	0.01841
DLX6	-2.19564	0.00030	1.10459	0.04308
EGR2	-1.17858	0.00133	1.23098	0.00217
EGR3	n.s.	n.s.	1.59588	0.00035
OTX1	n.s.	n.s.	3.65454	0.00035
OTX2	-1.73890	0.03221	1.24404	0.03936
PAX1	n.s.	n.s.	1.92862	0.00031
PAX2	n.s.	n.s.	2.09194	0.00424
PAX3	n.s.	n.s.	1.75708	0.00055
PAX4	n.s.	n.s.	2.89886	0.00146
PAX6	n.s.	n.s.	1.80547	0.00000
PAX7	-1.29488	0.03177	1.85923	0.00050
POU1F1	n.s.	n.s.	1.89961	0.02935
POU2AF1	n.s.	n.s.	2.04066	0.00205
POU2F2	n.s.	n.s.	0.99116	0.01655
POU2F3	n.s.	n.s.	3.00384	0.00018
POU3F2	-1.92369	0.00456	1.02421	0.01311
POU4F2	n.s.	n.s.	2.25746	0.00162
POU6F2	-1.62602	0.00603	1.11727	0.01458
PRDM7	n.s.	n.s.	2.17865	0.00030
PRDM9	-1.17279	0.00828	1.68343	0.00138

Continued table.

Gene ID	$log_2FC^{HIR/LIR}$	$FDR^{HIR/LIR}$	$log_2FC^{GnRHa+/-}$	$FDR^{GnRHa+/-}$
PRDM12	n.s.	n.s.	2.11445	0.01114
PRDM16	n.s.	n.s.	1.14955	0.00420
RUNX1	-0.95564	0.00848	0.75495	0.00369
RUNX2	n.s.	n.s.	1.22941	0.00296
SIX2	-1.76080	0.00941	1.61456	0.01446
SIX3	n.s.	n.s.	2.28491	0.00392
SOX1	-1.65838	0.00346	1.24834	0.01547
SOX2	n.s.	n.s.	1.24700	0.02304
SOX11	n.s.	n.s.	0.74816	0.00565
SOX14	n.s.	n.s.	3.95870	0.00052
SOX30	-1.94278	0.00025	0.72938	0.04419

Abbreviations: HIR – high infertility risk subgroup samples; LIR – low infertility risk subgroup samples; GnRHa- – 2HIR subgroup samples before hormonal treatment, GnRHa+ – after hormonal treatment; log_2FC – binary logarithm of fold-change; FDR – false discovery rate; n.s. – not significant.

3.4. Expression of lncRNAs in cryptorchid testes and GnRHa effects on their expression (Hadziselimovic *et al.*, 2017, 2019)

Preliminary differential gene expression analysis disclosed that adjuvant GnRHa treatment increased the expression of some antisense and non-coding RNAs involved in:

- embryogenesis: *FENDRR* (Grote and Herrmann, 2013; Dey, Mueller and Dutta, 2014);
- X chromosome inactivation (XCI) process: *TSIX* (Lyon, 1961);

- histone modifications: *AIRN*, *HOTAIR* (Nagano *et al.*, 2008; Gupta *et al.*, 2010).

Based on initial results, a targeted expression analysis of lncRNAs was performed. First, lncRNAs with differential expression in HIR and LIR samples were determined. Next, a comparison of the second research group samples was executed: changes in gene expression before (Surgery 1) versus after (Surgery 2) surgical treatment (1HIR subgroup) and before (GnRHa-) versus after (GnRHa+) adjuvant hormonal therapy (2HIR subgroup) were determined. Results showed a positive GnRHa effect on the expression of lncRNAs. On the contrary, surgical treatment alone had no significant impact on inducing changes in their expression.

The dataset was explored with Volcano plots to detect genes with biggest (log_2FC) and significant (FDR) changes in the expression levels between HIR and LIR subgroups, and before versus after GnRHa treatment (2HIR subgroup samples). It was found that 627 lncRNAs exhibited decreased expression in the HIR subgroup compared to LIR, whilst 38 exhibited increased expression (Figure 7A). A comparison of samples from the 2HIR subgroup displayed an increased expression of 3 074 lncRNAs, and a decreased expression of 53 (Figure 7B).

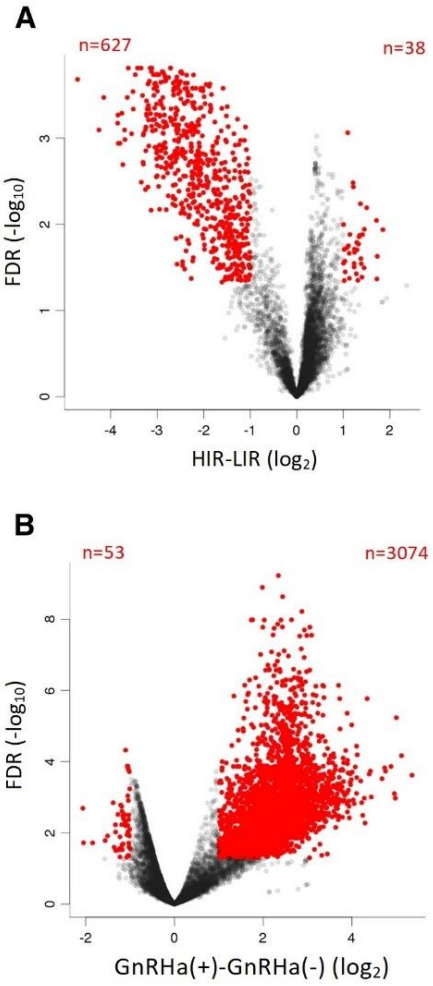


Figure 7. *Volcano plots of lncRNA expression in cryptorchid testes:* A – between high (HIR) and low (LIR) infertility risk subgroups; B – in 2HIR subgroup before (GnRHa(-)) and after (GnRHa(+)) adjuvant hormonal treatment. Genes with no significant difference between subgroups are in black. Differentially expressed genes are in red. The most upregulated and downregulated genes are on the right and on the left, respectively. Most significant genes are at the top (Hadziselimovic *et al.*, 2019).

The potential roles of lncRNAs, responsive to GnRHa treatment, were identified based on their genomic location, association with protein coding genes in sense / antisense pairs, RNA-RNA interactions and data provided in the literature. Eleven of 77 lincRNAs and four of 46 AS-lncRNAs with FC > 2 following hormonal treatment were selected (Table 6). The pattern of changes in expression induced by GnRHa enabled to hypothesize that these selected lncRNAs are important for Ad spermatogonia development, are involved in stem cell renewal, signaling, cell differentiation, morphogenesis and genome stability. Moreover, seven lncRNAs with decreased expression in HIR subgroup and GnRHa-induced that are linked to cancer development and transition of Ad spermatogonia were selected: *LINC00221*, *LINC00701*, *LINC00922*, *LINC01249*, *LINC01446*, *HOTAIR*, *DLX6-AS1*.

Analysis showed that adjuvant GnRHa treatment has no significant impact on expression of downregulated *TINCR* (*Terminal differentiation-induced non-coding RNA*) in HIR testes ($\log_2$FC = -1.07; FDR = 0.002). In contrast, GnRHa suppressed the expression of epigenetic factors upregulated in the HIR subgroup that bind TINCR (Gong *et al.*, 2018): *ARID4B*, *ARID5B*, *BPTF*, *CHD6*, *KDM6A*, *MBD2*, *SETD7* (Table 7).

Table 6. *Testicular lincRNAs and AS-lncRNAs involved in stem cell renewal and differentiation, upregulated by GnRHa* (Hadziselimovic *et al.*, 2019)

Gene ID	RPKM GnRHa- Median / MAD	RPKM GnRHa+ Median / MAD	$log_2FC^{GnRHa+/-}$	p value	FDR
LINC-ROR	0.044 / 0.06	0.40 / 0.24	2.68	0.0004	0.002
LINC00261	0.11 / 0.04	1.11 / 0.68	2.60	3.588E-08	4.35E-06
LINC00293	0.05 / 0.04	0.66 / 0.49	2.80	0.0001	0.001
LINC00303	0.22 / 0.07	1.25 / 0.45	2.57	0.0001	0.0008
LINC00520	0.15 / 0.07	0.79 / 0.64	2.76	0.0002	0.001
LINC00898	0.04 / 0.03	0.35 / 0.25	2.72	0.0002	0.001
LINC00974	0.07 / 0.04	0.48 / 0.46	3.9	0.0008	0.003
LINC00994	0.20 / 0.10	1.41 / 0.81	2.73	5.773E-06	0.0001
LINC01016	0.15 / 0.03	1.32 / 0.70	3.36	2.078E-09	6.43E-07

Gene ID	RPKM GnRHa- Median / MAD	RPKM GnRHa+ Median / MAD	$log_2FC^{GnRHa+/-}$	p value	FDR
LINC01121	0.25 / 0.04	1.84 / 0.45	2.89	2.049E-09	6.43E-07
LINC01553	0.09 / 0.06	1.26 / 0.67	3.60	0.0002	0.001
EGFR-AS1	0.03 / 0.05	0.58 / 0.30	2.99	3.850E-07	2.28E-05
HOTTIP	0.04 / 0.03	0.47 / 0.37	2.22	0.0010	0.004
mTOR-AS1	0.21 / 0.19	1.90 / 1.33	4.96	6.67E-06	0.0001
OTX2-AS1	0.09 / 0.007	0.91 / 0.45	2.37	2.95E-06	8.83E-05

Abbreviations: RPKM – reads per kilo base per million mapped reads; MAD – median absolute deviation; GnRHa- – 2HIR subgroup samples before hormonal treatment, GnRHa+ – after hormonal treatment; log_2FC – binary logarithm of fold-change; FDR – false discovery rate.

Table 7. *Expression of epigenetic factors in undescended testes that bind TINCR* (Hadziselimovic *et al.*, 2019)

Gene ID	**$log_2FC^{HIR/LIR}$**	**$FDR^{HIR/LIR}$**	**$log_2FC^{GnRHa+/-}$**	**$FDR^{GnRHa+/-}$**
ARID4B	+0.18	0.041	-0.63	0.008
ARID5B	+0.35	0.004	-0.49	0.003
BPTF	+0.19	0.055	-0.61	0.01
CHD6	+0.21	0.040	-0.54	0.03
KDM6A	+0.20	0.017	-0.81	0.0009
MBD2	+0.23	0.029	-0.68	0.004
SETD7	+0.22	0.042	-0.85	0.0006

Abbreviations: HIR – high infertility risk subgroup samples; LIR – low infertility risk subgroup samples; GnRHa- – 2HIR subgroup samples before hormonal treatment, GnRHa+ – after hormonal treatment; log_2FC – binary logarithm of fold-change; FDR – false discovery rate.

CONCLUSIONS

1. Adjuvant hormonal treatment with GnRHa induces gonocyte transition to Ad spermatogonia.
2. Differential gene expression in undescended testes significantly differs between high and low infertility risk groups: in most cases genes in HIR are downregulated.
3. Adjuvant hormonal treatment with GnRHa stimulates the expression of genes involved in hormonal control of HPG axis and fertility, affects loci involved in canonical and altenative testosterone synthesis pathways.
4. Determined non-coding RNAs play an important role in the regulation of spermatogenesis acting at early stages of spermatogonial stem cell development and respond to GnRHa stimulation.

RECOMMENDATIONS

1. An assesment of infertility risk in cryptorchidism is advocated – a histological evaluation of a testicular biopsy obtained during orchidopexy determining Ad spermatogonia and TFI is recommended.
2. Adjuvant hormonal treatment with GnRHa is recommended for cryptorchid boys with a histologically confirmed high infertility risk.
3. Further prospective studies with GnRH treatment before surgery in cryptorchid boys during mini-puberty and the execution of RNA sequencing and immunohistochemistry in testicular biopsies are advocated to confirm hypotheses.

PUBLICATIONS AND PRESENTATIONS

1. Publications

- Vincel B, Verkauskas G, Bilius V, Dasevicius D, Malcius D, Jones B, Hadziselimovic F. Gonadotropin-releasing hormone agonist corrects defective mini-puberty in boys with cryptorchidism: a prospective randomized study. *Biomed Res Int*. vol. 2018, Article ID 4651218, 5 pages, 2018. https://doi.org/10.1155/2018/4651218
- Hadziselimovic F, Verkauskas G, Vincel B, Stadler MB. Testicular expression of long non–coding RNAs is affected by curative GnRHa treatment of cryptorchidism. *Basic Clin Androl*. 2019 Dec 27;29:18. doi: 10.1186/s12610-019-0097-3

2. Publications partly related to the topic of dissertation

- Adomaitis R, Vincel B, Eidukaite A, et al. Consequences of bilateral cryptorchidism in adults. *Andrologia*. 2016;48(9):933-938. doi:10.1111/and.12534
- Hadziselimovic F, Verkauskas G, Vincel B, Krey G, Zachariou Z. Abnormal histology in testis from prepubertal boys with monorchidism. *Basic Clin Androl*. 30, 11 (2020). https://doi.org/10.1186/s12610-020-00109-1

3. Presentations

- Vincel B, Verkauskas G, Bilius V, Dasevičius D, Malcius D, Hadziselimovič F. Prospective randomized study of hormal treatment in boys with bilateral cryptorchidism. EUPSA 2017. May 17-20, 2017. Limassol, Cyprus.
- Hadziselimovič F, Verkauskas G, Vincel B, Stadler MB. Epigenetic code of long non-coding RNAs after curative GnRHa

treatment for cryptorchidism-induced infertilityx. EUPSA 2019. June 12-15, 2019. Belgrade, Serbia.

4. Presentations partly related to the topic of dissertation

- Vincel B, Geimanaitė L, Dasevičius D, Verkauskas G. Histological evaluation of gonads in patients with disorders of sex development. 15th Conference of the Baltic Association of Paediatric Surgeons. May 10-12, 2018. Riga, Latvia.
- Vincel B, Dasevičius D, Bilius V, Puzinas A, Ivanauskienė D, Verkauskas G. Histological evaluation of gonads in patients with disorders of sex development. 8th Warsaw Conference of Paediatric Urology. September 6-8, 2018. Radziejovice, Poland.
- Hadziselimovič F, Verkauskas G, Vincel B, Stadler MB. Cryptorchid boys with abrogated mini-puberty display differentially expressed genes involved in sudden infant death syndrome The 58th Annual ESPE Meeting. September 19-21, 2019. Vienna, Austria.

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Michael B. Stadler, Friedrich Miescher Institute for Biomedical Research, for bioinformatic research and statistical analysis of molecular data.

CURRICULUM VITAE

Personal information

BEATA VINCEL, born 1985-06-05

Address: 56-5 Stanevičiaus Str., Vilnius 07117, Lihuania

Phone number: +370 6 7439320

Email address: *beata.vincel@gmail.com*

Education

2016–2020	Vilnius University, Faculty of Medicine, Doctoral (PhD) studies
2010–2015	Vilnius University, Faculty of Medicine, Residency in Paediatric surgery
2004–2010	Vilnius University, Faculty of Medicine, Medicine
2008–2009	University of Kuopio, Medicine (Erasmus programme)
2000–2004	Vilnius Adomo Mickevičiaus Gymnasium

Work experience

2014-05–2016-01	Medical doctor, Beauty Therapy and Surgery Clinic Sugihara

2014-08–2015-09	Medical doctor, Children's Hospital, Emergency Department
2016-11–2017-01	Paediatric surgeon; Public Institution Centro Poliklinika
2015-08–present	Paediatric surgeon, Vilnius University Hospital Santaros Klinikos, Children's Surgery Department

Professional qualifications

2008 July 1–29	The Infant Jesus Teaching Hospital (Szpital Kliniczny Dzieciątka Jezus), Department of General Surgery and Transplantology, Warsaw, Poland
2009 July 3–31	City hospital No.35 (Больница №35 городская МЛПУ), Department of Anesthesiology and Intensive Care, Nizhny Novgorod, Russia
2010 March 6	Crisis Research Center, "Compulsory First Aid Training Program," Kaunas, Lithuania
From 2013	Annual Conference of the Lithuanian Society of Pediatric Surgeons
2013 May 17–19	Crisis Research Center, professional qualification course "Emergency Medical Care for Children," Kaunas, Lithuania
2013 November 14–15	EPOS Regional Core Curriculum Courses, Kaunas, Lithuania

2014 June 18–21	15th European Pediatric Surgeons' Association Congress (EUPSA 2014), Dublin, Ireland
2014 June 18	Basic Skills in Paediatric Laparoscopy Course, EUPSA 2014, Dublin, Ireland
2014 September 25–27	XIII Conference of the Baltic Association of Paediatric Surgeons (BAOPS 2014), Vilnius, Lithuania
2014 November 2–30	V. Buzzi Childrens Hospital (Ospedale dei Bambini V. Buzzi), Department of Pediatric Surgery, Milan, Italy
2015 March 20–21	8th Central & Eastern European Course in Pediatric Surgery, Master Course in Advanced Pediatric Minimal Access Surgery, Szeged, Hungary
2016 May 5–7	XIV Conference of the Baltic Association of Paediatric Surgeons (BAOPS 2016), Tartu, Estonia
2016 October 17–21	"General Competence Skills Training," Vilnius, Lithuania
2017 May 17–20	18th European Pediatric Surgeons' Association Congress (EUPSA 2017), Limassol, Cyprus
2018 April 11–14	29th Congress of ESPU, Helsinki, Finland
2018 May 10–12	15th Conference of the Baltic Association of Paediatric Surgeons (BAOPS 2018), Riga, Latvia

2018 June 20–23	19th European Pediatric Surgeons' Association Congress (EUPSA 2018), Paris, France
2018 September 6–8	8th Warsaw Conference of Pediatric Urology, Radziejovice, Poland
2019 March 20–23	12th Central and Eastern European Course in Pediatric Surgery, Minimally Invasive and Neonatal Surgery, Lviv, Ukraine
2019 April 24–27	30th Congress of ESPU, Lyon, France
2019 June 12–15	20th European Pediatric Surgeons' Association Congress (EUPSA 2019), Belgrade, Serbia
2019 December 5–6	12th Annual European Pediatric Colorectal and Pelvic Reconstruction Congress & 7th Paediatric Surgery Postgraduate Seminars, Vienna, Austria
2019 December 7	The PSARP- and Laparoscopic Training Workshop, Vienna, Austria

Good Clinical Practice

1. Good Clinical Practice Course. Inlita. 2016 July 19. Certificate No. IL/C.03.16
2. Good Clinical Practice: A Refresher Course for all Site Personnel Working on Clinical Research Studies (including ICH E6, Revision 2 Changes) Version 2, MARCH 2017, 2017 October 4

Presentations

"Liver rupture treatment in children using a stent graft." 15th EUPSA

2014 Congress, Dublin, Ireland.

"Consequences of bilateral cryptorchidism in adults." 14th Conference of the Baltic Association of Paediatric Surgeons (BAOPS 2016), Tartu, Estonia.

"Consequences of bilateral cryptorchidism in adults." 3rd EAU Baltic Meeting (BALTIC16), Tallinn, Estonia (co-author).

"Cryptorchid boys with abrogated mini-puberty display differentially expressed genes involved in sudden infant death syndrome." 58th Annual ESPE Meeting, Vienna, Austria (co-author).

"Acute appendicitis in the newborn: clinical cases and literature review." Annual Conference of the Lithuanian Society of Pediatric Surgeons 2019, Kaunas, Lithuania (co-author).

Memberships

2015–present	Member of the Lithuanian Society of Pediatric Surgeons
2018–present	Member of the European Society for Paediatric Urology (ESPU)

Areas of interest

Cryptorchidism, fertility prognosis, disorders of sex development (DSDs), minimally invasive pediatric surgery.

NOTES

Vilnius University Press
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