Transnational University Limburg is a unique collaboration of two universities in two countries: the University of Hasselt and Maastricht University.

UHASSELT
KNOWLEDGE IN ACTION

www.uhasselt.be
Universiteit Hasselt
Campus Hasselt:
Martelarenlaan 42 | 3500 Hasselt
Campus Diepenbeek:
Agoralaan Gebouw D | 3590 Diepenbeek

UHASSELT
KNOWLEDGE IN ACTION

Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

The optimisation of RNA extraction from corpus cavernosum for the analysis of physiological pathways involved in erectile dysfunction

Lore Raets

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Clinical Molecular Sciences

SUPERVISOR :
dr. Kimberly VANHEES

SUPERVISOR :
Prof. Dr. Koenraad VAN RENTERGHEM

MENTOR :
Mevrouw Benedith OBEN

2018
2019

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

The optimisation of RNA extraction from corpus cavernosum for the analysis of physiological pathways involved in erectile dysfunction

Lore Raets

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Clinical Molecular Sciences

SUPERVISOR :

dr. Kimberly VANHEES

SUPERVISOR :

Prof. Dr. Koenraad VAN RENTERGHEM

MENTOR :

Mevrouw Benedith OBEN

Acknowledgements

First of all, I want to thank Professor Dr. Van Renterghem for the opportunity to perform this internship at the Jessa Hospital. During this internship, he gave me the freedom to develop my research skills. In addition, I had the chance to join Prof. Dr. Van Renterghem to the ESSM congress in Ljubljana, Slovenia. This was a major experience, where I learned a lot about erectile dysfunction. Furthermore, he always supported my decisions and gave me good advice how to conduct my thesis research.

Next on, I want to thank dr. Kimberly Vanhees to become my promotor during this internship. You were always there with your support and good advice. Thank you for the many times I dropped in your office with questions and you still took the time to listen to them.

Thank you Bénedith Oben, for the countless times I asked your opinion on my thesis or on my lab work. Thank you for helping me in the optimisation of my protocols and for teaching me how to conduct these protocols in the best way possible. Thank you for sharing the haematology/biobank lab of Jessa Hospital with me.

Dr. Patrick Vandormael, as my second examiner, you gave me great advice in how to tackle my research hypothesis and especially the difficulty in extracting descent RNA from my samples. You also were so kind to share your experience in RNA extraction and gave me the opportunity to perform extractions in the Biomed lab. Thank you, dr. Ingrid Arijs, for the help during the start of my master thesis and the support and advice during RNAsequencing.

I also want to thank Evi Vanoppen. The countless times I mailed you to obtain corpus cavernosum samples from the Biobank for me… I am very thankful you were there for this processing. This is also where I want to thank the UBiLim Biobank in general, you all do an amazing job. Samples are perfectly stored and the documentation was always accurate.

An important group of girls I want to thank is our senior group. Lene, Victoria, Sofie and Lieselotte, thank you for the laughs and tears we had together. We always had each other's back. Thank you for the countless times we laughed about the most ridiculous stuff and the great conversations in good and bad times. Furthermore, I want to thank everyone from "Huis81". Thank you for involving us in the activities, for the laughter and the countless times we had cake together. Thank you all for always being there to support me with advice for my thesis.

Lastly, but not the least, I want to thank my family and boyfriend. Thank you, mom, dad and my brother Cedric for the support and for the opportunity to start this master and finish it successfully. Thank you, Laurens, for always listening to me and having my back in every decision I made in the past months. Thank you all for always showing me how proud you all were and how much you all wanted me to succeed in this master thesis. To conclude, thank you Leen, for the journey called "schakeljaar" we successfully travelled together the past three years.

Thank you!

Lore Raets

Table of contents

Abbreviations

AML - acute myeloid leukaemia

ANGPTL4 - angiopoietin-like 4

BMI – body mass index

Ca^{2+} - calcium

CBS - cystathionine β-synthase

CC – corpus cavernosum

cDNA – copy DNA

cGMP - cyclic guanosine monophosphate

CSE - cystathionin γ-lyase

Ct – cycle threshold

dCt – delta cycle threshold

ED – erectile dysfunction

eNOS - endothelial NO synthase

GTP – Guanosine triphosphate

HBB - haemoglobin subunit beta

HMBS - hydroxymethylbilane synthase

H_2S – hydrogen sulphide

IIEF – international index of erectile function

IPP - inflatable penile prosthesis implantation

IQR - interquartile range

K^+ - potassium

KANSL1-AS1 - KAT8 regulatory NSL complex subunit 1-antisense RNA 1

L-cys - L-cysteine

NGS – next generation sequencing

nNOS – neuronal NO synthase

NO – nitric oxide

NTC – non-template control

OD – optical density

PDE5-I – phosphodiesterase type 5 inhibitors

PGE1 – prostaglandin 1

QC – quality control

qPCR – quantitative polymerase chain reaction

RIN - RNA integrity number

RLP9 - ribosomal protein L9

RNA – ribonucleic acid

RNAseq – RNA sequencing

ROS – reactive oxygen species

RT – room temperature

SEP – sexual encounter profile

sGC - soluble guanylyl cyclase

SPSS - Statistical Package for the Social Sciences

TRT – testosterone replacement therapy

UBiLim – university biobank Limburg

Abstract

Introduction - Erectile dysfunction (ED), defined as the inability to attain or maintain a penile erection for sexual intercourse, can be caused by a severe medical condition (e.g. diabetes or prostate cancer treatment). Currently, 40% of ED patients do not respond to non-invasive treatment (i.e. Viagra). They are assigned to more invasive options, such as inflatable penile prosthesis (IPP) surgery. Therefore, the search for new potential therapeutic targets to help these refractory ED patients is highly required. However, before this search can start, good quality of corpus cavernosum (CC) RNA must be provided. Therefore, we aim to optimise RNA extraction from biobank-stored CC, which have never been done before according to our knowledge.

Hypothesis - The biobank-stored CC samples are still of sufficient quality and quantity for downstream applications and further analysis of the pathophysiological pathways involved in ED.

Materials & methods – The CC tissue samples were obtained from patients who had IPP surgery. Refractory ED patients were patients with diabetes, vascular dysfunctions, hypogonadism, Peyronie's disease or radical prostatectomy. The tissue was collected in RNA*later* solution or snap frozen. For CC homogenisation, the GentleMACS and pestle were compared. Different RNA extraction protocols, i.e. QIAzol method and RNeasy mini kit, were optimised. RNA quality, quantity and integrity were studied with the NanoDrop and Bioanalyzer. RNA was transcribed into cDNA using the SuperScript™ IV First-Strand Synthesis System. Additionally, a duplex quantitative polymerase chain reaction (qPCR) was performed with hydroxymethylbilane synthase (*HMBS)* and haemoglobin subunit beta (*HBB*) to determine the quantity and quality of the cDNA samples. Lastly, six samples were sent to GenomicsCore, Leuven to perform a Quantseq analysis. Statistical significance (p<0.05) was evaluated with Mann-Whitney U or independent samples T-Test.

Results - Different RNA extraction methods showed that the CC tissue was optimal homogenised using the GentleMACS. In contrast to the manual pestle, this device is a mechanical method, with enough force to disrupt the tissue. RNA*later* CC tissue samples with GentleMACS homogenisation and RNeasy mini kit extraction provided the highest quantitative and qualitative RNA. Two different collection methods were tested during optimisation, RNA*later* and snap frozen. When comparing RNA*later* and snap frozen samples, no significant differences in concentration, OD values and RNA integrity number (RIN) were shown. Despite no significant differences, RNA*later* samples gave higher RIN values compared to snap frozen tissue. Furthermore, there was no significant correlation between time until processing and RNA concentration or OD values. RNA quality and quality were sufficient to perform a duplex qPCR for the *HMBS gene* and *HBB gene.* RNA quality was even sufficient to perform RNAseq. Moreover, three genes, *ANGPTL4, RPL9* and *KANSL1-AS1* were found to be significantly differently expressed between the vascular and diabetic group.

Discussion & conclusion – RNA isolated from biobank-stored CC samples is still of sufficient quality and quantity to be used for downstream applications, such as qPCR and RNAseq. Homogenization of RNA*later* stored CC tissue using the GentleMACS followed by RNA extraction using the RNeasy mini kit has the best result. RNA obtained via this method can be used in Quantseq analysis for differential gene expression. Furthermore, cDNA can be produced from this RNA to perform qPCR. The low QC values of the performed qPCR again proved the high quality of the isolated RNA.

1. Introduction

1.1 Erectile dysfunction

Erectile dysfunction (ED) is defined as the inability to attain or maintain a penile erection sufficient for sexual intercourse (1). Men can develop ED for many reasons, for example due to relationship problems or lifestyle habits (i.e. smoking, no exercise) (2). However, there are also patients who suffer from a severe medical condition (i.e. diabetes, prostate cancer treatment) leading to ED. This dysfunction has a significant effect on patients' sex life and psychological well-being (3). The patients' self-esteem will decrease and the quality of his (sexual) relationship will worsen (4). This dysfunction mostly affects men over the age of 40 (1). The prevalence of ED increases from 14% in 41-year-old-men to 41% in 80-year-old-men (5, 6). Since the population is growing older, ED is becoming a major health problem (1, 7). The corpus cavernosa (CC), expandable erectile tissues of the penis, are key players in the development of ED.

1.2 Anatomy of the penis

The penis consists of two CC and one ventral corpus spongiosum, which contains the urethra (**Figure 1**). The CCs consists of endothelial-lined vascular spaces called sinusoids. These sinusoids are composed of a mesh of trabeculae, consisting of smooth muscle, collagen, and a complex array of arterioles and nerves (8). CC are surrounded by a strong fibrous envelope, the tunica albuginea. CC itself are highly trabeculated with fibroelastic and muscle fibres. During penile erection, the trabecular smooth muscle relaxation increases the compliance of the sinusoids. This causes them to expand to accommodate the increased blood flow (8). Furthermore, the penis has enveloping layers of fascia, nerves, lymphatics, blood vessels and skin (8).

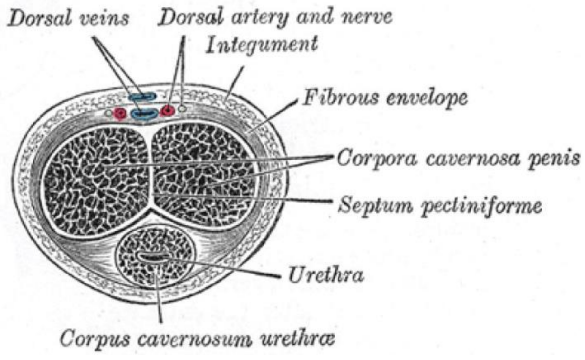


Figure 1: Anatomy of the penis
The penis consists of two corpora cavernosa (CC), which are surrounded by a strong fibrous envelope, called the tunica albuginea. The penis has enveloping layers of fascia, nerves, lymphatics, blood vessels and skin

Stability of the penis is ensured by the penile ligaments (fundiform ligament and suspensory ligaments). The penis is attached to the pubic symphysis and the linea alba of the rectus sheath (8).

Three penile arteries are formed out of the internal pudendal artery: bulbourethral artery (which supplies the corpus spongiosum and the urethra), the deep bilateral penile (cavernosal) artery (which consists of helicine arteries that are important for the erectile process), and the dorsal penile artery (which supplies the glans, prepuce, fascia and skin). The venous system consists of a superficial, intermediate and deep drainage system (8).

Autonomic control is provided out of the pudental nerve and the pelvic plexus. The autonomic nerve fibres innervate the helicine arteries. The cavernous trabeculae contain the adrenergic nerve fibres and receptors. They surround the deep penile arteries. Two important neurotransmitters are involved

in the controlling of penile erection, with noradrenaline as the main neurotransmitter controlling penile flaccidity and tumescence, and nitric oxide (NO) for the penile erection (8).

The CC play a key role in erection and thus ED. Because there are no alterations in the anatomy of the CC of ED patients, the underlying physiology must play an important role in ED.

1.3 Physiology of penile erection

1.3.1 Nitric oxide-soluble guanylyl cyclase-cyclic guanosine monophosphate pathway

The most important pathway involved in penile erection is the NO-soluble guanylyl cyclase (sGC)-cyclic guanosine monophosphate (cGMP) pathway (**Figure 2**) (1, 9). This pathway is based on the known key role of NO as a neurotransmitter for smooth muscle cell relaxation in CC. Sexual arousal leads to an increased blood flow to the CC of the penis. By this increased blood flow, the amino acid L-Arginine is activated. L-Arginine and molecular oxygen generate NO via a reaction catalysed by endothelial NO synthase (eNOS) (9). NO is released from endothelial cells. Additionally, NO is also released from the cavernous nerves, when they are depolarised (9). This NO release increases intracellular Calcium (Ca^{2+}) concentrations, which binds to cytosolic protein calmodulim (Cam) and activates NOS in cavernous nerves (nNOS) (9). Additionally, NO diffuses to the smooth muscle cells of the CC, where it will enter the smooth muscle cells.

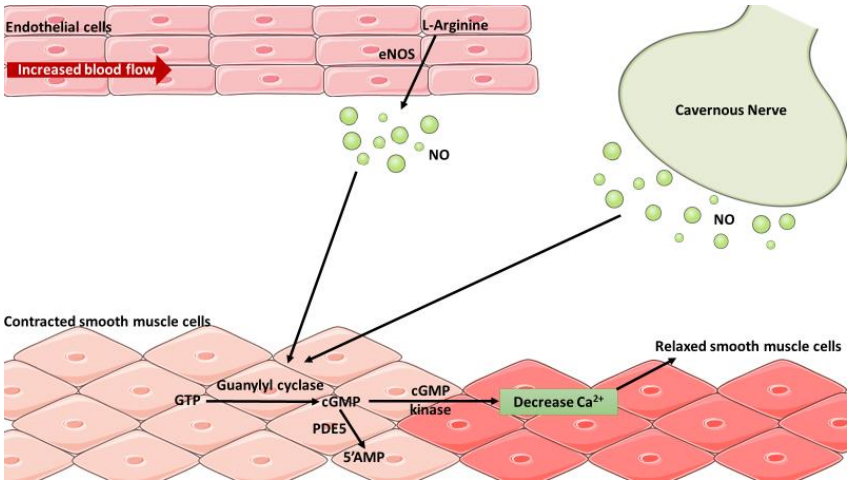


Figure 2: Nitric oxide (NO)-soluble guanylyl cyclase (sGC)-cyclic guanosine monophosphate (cGMP) pathway in the corpus cavernosum (CC).
The different parts of the NO sGC cGMP pathway involved in first line therapy are highlighted in this figure. Endothelial cells and cavernous nerves release NO, which is the main neurotransmitter of the NO-sGC-cGMP-pathway. A sufficient concentration of cGMP is required for penile erection. Smooth muscle cells will relax which is necessary for penile erection. Phosphodiesterase 5 (PDE5) is an enzyme which degrades intracellular cGMP. This is not desirable, because cGMP concentration will lower, and penile erection will not occur. Based on Shamloul et al. (1)

NO activates sGC in the cavernous smooth muscle cells to generate cGMP from intracellular guanosine triphosphate (GTP) (1, 9, 10). This production of cGMP activates a cGMP-dependent protein kinase, causing membrane hyperpolarisation through potassium (K^+) channels in the smooth muscle cell membrane and the increase in uptake of Ca^{2+} into the endoplasmic reticulum (10). Smooth muscle cell relaxation occurs through this hyperpolarisation and the blockade of membrane Ca^{2+} channels, which decreases intracellular Ca^{2+} (10).

1.3.2 Alternative pathways

Beside the NO-pathway, other pathways can induce a penile erection. A second pathway known for its ability to cause relaxation in CC, is the hydrogen sulphide (H_2S)-pathway. Via this pathway, H_2S can relax the smooth muscle cells of CC (11). H_2S is a neurotransmitter that is endogenously produced from L-cysteine (L-cys) by the activity of two enzymes: cystathionine β-synthase (CBS) and cystathionin γ-lyase (CSE) (11). The exact mechanism of H_2S on the CC is not yet known. However, it is proposed that H_2S has an effect on the K^+ channels. The effects of H_2S, acting like a regulatory mediator, are similar to the relaxation effects of NO. Previous organ bath studies showed that H_2S has a relaxant effect independent of the endothelium, which is favourable for patients with ED, since the endothelium is often compromised in ED patients (11, 12).

Another important pathway for penile erection is the RhoA/Rho-kinase pathway. RhoA activates Rho-kinase. This activated Rho-kinase phosphorylates the regulatory subunit of smooth muscle myosin phosphatase and prevents dephosphorylation of myofilaments (13, 14). This mechanism will lead to a contractile tone of CC tissue (13, 15, 16). However, penile erection requires relaxation of CC tissue. Therefore, an abnormal enhanced RhoA/Rho-kinase pathway activity is linked to ED (9). Selective inhibition of Rho-kinase, for example through cGMP kinases, evokes relaxation of the CC (9, 13, 15). This makes Rho-kinases a possible therapeutically target for patients with ED (17).

Reactive oxygens species (ROS) are also responsible for a reduced NO availability and an increased apoptosis of both endothelial and neuronal cells (16, 18).

1.4 Pathophysiology

The inability to attain or maintain a penile erection consists of a combination of psychogenic, neurogenic, endocrinological and vasculogenic systems. These systems contribute to the changes in physiological pathways mentioned earlier. They often have an effect on the NO-pathway.

Psychogenic factors have a large contribution to ED. One important example is performance anxiety (1). Stress and depressive behaviour can also influence ED in a negative way, because noradrenaline is known as an anti-erectile neurotransmitter (19). Examples of neurogenic causes of ED are multiple sclerosis, spinal cord injury, Parkinson disease and radical prostatectomy. Sacral lesions induce structural and functional alterations due to the decreased innervation (19). Because nerves are damaged, the NO concentration will be reduced and relaxation of smooth muscle cells will not occur, compromising penile erection. Lowering NO concentrations have an influence on the NO-pathway, reducing penile erection. An important cause of endocrinological ED is hypogonadism. These patients have a lower concentration of testosterone (19), which is important in the formation of NOS and PDE5 in the penis, having again an impairment of the NO-pathway (1). Besides lower testosterone concentrations, there is also an altered expression of connexin 43, which can also be a cause of ED in these patients (20). Changes in vasculogenic systems can affect ED too. Endothelial dysfunction contributes to the pathophysiology of ED by a decreased release of NO. Vasculogenic causes of ED are atherosclerosis, hypertension, hyperlipidaemia, cigarette smoking, diabetes mellitus, and pelvic irradiation, which all have an effect on the NO-pathway (1, 21). Moreover, ED can be a strong predictor for cardiovascular disease, in particular coronary artery disease (1).

However, beside the previous mentioned causes, ED can also be drug-induced. Psychotropic drugs, such as antidepressants are the most common drugs that can lead to ED. Furthermore, antihypertensive drugs, such as thiazides and β-blockers, can also induce ED. These drugs increase the intracellular Ca^{2+} concentrations, which will lead to contraction instead of relaxation. However, it is not certain whether ED is caused by the medication itself or the underlying disease (i.e. hypertension) (19). Moreover, narcotic and alcohol abuse can contribute to the development of ED (1, 21).

Lastly, ageing, lifestyle factors and systemic diseases can induce ED. (1). Two large-scale studies confirmed the link between increased age and increased prevalence and severity of ED (22, 23). After age, diabetes mellitus type 2 is the second most common risk factor for ED. Diabetes mellitus type 2 occurs three times more in diabetic males compared to non-diabetic males and develops in 50-75% of diabetic males (1, 24, 25). Evidence showed that sedentary lifestyle, smoking, alcohol or drug abuse, sleep disorders, obesity, and metabolic syndromes all can contribute to ED (1, 26-28). Finally, chronic kidney, liver, and pulmonary diseases have been associated with ED (1, 29-31).

The effect of these pathophysiological causes is often explained via the NO-pathway. However, the exact role of other genes and pathways is not fully understood. For this reason, transcriptomics could reveal new links between these pathophysiological causes and pathways that are known to contribute to penile erection. Transcriptomics focusses on the transcriptome of the human tissue, more precisely the total ribonucleic acid (RNA) in the CC of ED patients. By investigating this transcriptome, differential gene expression can be measured. Dysregulated genes can also be coupled to specific pathways.

Little research was done in transcriptomic analysis of erectile dysfunction. Searching through PubMed showed less than 30 research articles concerning genetic analysis in ED. Much research was done in animal models, so there is a need of performing these analyses on human samples. To our knowledge, next generation sequencing (NGS) was never used for transcriptomic analysis, but it will provide new insights on the pathophysiology of ED (32).

1.5 Diagnosis

Diagnosis of ED patients start with a detailed medical and sexual history of the patient (**Appendix Figure 1**). When available, the partner's medical and sexual history is also taken in account. This history exchange can reveal common disorders that contribute to ED (33). This conversation must include information about sexual orientation, previous and current sexual relationships, current emotional status, onset and duration of the erectile problem, and previous consultations and treatments (21). Furthermore, the rigidity and duration of both sexually-stimulated and morning erections should be described in detail. Problems with sexual desire, arousal, ejaculation, and orgasm are also of great importance (34, 35). Validated questionnaires such as the International Index for Erectile Function (IIEF) and the sexual encounter profile (SEP) question-2 (SEP-2) and question-3 (SEP-3) (36, 37) can be used to assess the different aspects of sexual history (i.e. sexual desire, erectile function, orgasmic function, intercourse, and overall satisfaction) and sexual domains (21).

Because depression can also be a cause of ED, it is important to inform on the psychological well-being of the patient with the use of validated questionnaires.

Besides questionnaires and conversations about the history of the patient, a physical examination is required. This examination focus on the genitourinary, endocrine, vascular and neurological systems (38, 39). This can possibly reveal undiagnosed diseases that cause ED, such as Peyronie's disease, pre-malignant or malignant genital lesions or signs and symptoms suggesting hypogonadism (small testes, alterations in secondary sexual characteristics etc.) (21). Furthermore, it is recommended to check blood pressure, heartrate and body mass index (BMI) of the patient (21).

Depending on the complaints and risk factors of the patient, laboratory testing will be performed. If not recently determined, a fasting blood glucose or haemoglobin type A1C (HbA1C) and lipid profile must be assessed. Furthermore, hormonal tests including an early morning total testosterone are checked (21).

Since some ED patients cannot be diagnosed with a sexual and medical history, there are also specialised diagnostic tests for ED. An example of such specialised diagnostic test is the pharmaco penile duplex ultrasonography (21). After intracavernosal injection with erection stimulating agents (i.e. Papaverine, phentolamine and prostaglandin PGE1) or oral vasoactive agents, the cavernosal arteries will be evaluated using the doppler technique. This is useful in the evaluation of vasculogenic causes (40, 41).

1.6 Treatment

There are considerable therapies for ED patients. Even though the dysfunction can be treated successfully, it is impossible to cure. Moreover, there are also refractory ED patients. Generally, there are three types of treatment: first-, second- and third-line therapy.

1.6.1 First-line treatment

First-line treatment is a non-invasive way to help ED patients. Phosphodiesterase type 5 inhibitors (PDE5-I's) are the leading first-line treatment. However, therapy and lifestyle modifications are also essential in ED treatment.

Psychosexual and couple therapy

Since ED has a major effect on the patients' psychological well-being and (sexual) relationship, counselling therapy is essential (3). Furthermore, counseling is also an important treatment when ED is caused in a psychogenic way, for example by stress (1). Counselling therapy promotes the recovery of sexual intimacy and satisfaction. Couples move towards acceptance and grief about sexual losses. They can also learn to commit to new ways of sexual experience, such as oral sex, masturbation, and partnered genital touch (42).

Lifestyle modifications

Smoking, alcohol consumption, obesity, and limited physical activity are lifestyle factors that often contribute to ED. Research shows that targeting these factors have a favourable effect on ED, especially in younger patients (1, 43). Data determined that 30 minutes of moderate-intensity

activity each day, weight loss in obese men, and switching to a healthier, Mediterranean diet improves ED outcomes (44-46). Other studies have also presented that smoking has a direct relationship with ED. Men have increased ED with greater numbers of cigarettes smoked or more years of smoking (17, 19, 47). Chronic alcohol abuse can affect the liver, which will lead to low levels of testosterone, contributing to ED (48, 49). The European Association of Urology (EAU) states that lifestyle changes and risk factor modification must be added to any ED treatment (19, 21).

Phosphodiesterase type 5 inhibitors

PDE5-I's are based on the known key role of NO. These inhibitors are the first line therapy for patients with ED. PDE5 is a key enzyme which degrades intracellular cGMP, impairing the penile erection. Therefore, PDE5-I will interfere with this degradation and cGMP will accumulate intracellularly, leading to a penile erection (1). However, PDE5-I depends on NO and therefore, still requires sexual stimulation(19).

Sildenafil citrate, also known as Viagra® (Pfizer, New York, USA,) is a selective PDE5-I (1, 9, 19). Other examples of PDE5-I are Tadalafil, Vardenafil and Avanafil. There is no double- or triple-blinded multicentre studies to compare which of these PDE5-I's are best to use. Choice depends on patient experience and the frequency of sexual intercourse (21).

Important disadvantages of PDE5-I's are the high cost and incomplete understanding of the mechanisms of action (1). As a result of this incomplete understanding, PDE5-I's should be used with care in some patients who are treated for high blood pressure (1).

Although PDE5-I therapy is a good therapy for 60% of the ED patients, still up to 40% of patients are poor to non-responders. These poor to non-responders often have impaired NO bioavailability, which is caused by other diseases.

Testosterone-deficiency has a negative impact on the working of PDE5-I. Testosterone-replacement therapy (TRT) helps to improve the working of PDE5-I in hypogonadism men (39). Because of the multifactorial pathophysiology of ED, also TRT will not be a suitable therapy for all ED patients. The combination of testosterone with PDE5-I can be a good therapy and may improve outcome (50). In conclusion, TRT is most suitable for hypogonadal men (51).

1.6.2 Second-line treatment

When first-line therapy does not work, second-line therapy is initiated. Intracavernosal injection and vacuum constrictive devices are the two main second-treatments used in ED.

Intracavernosal injection and transurethral therapy

Intracavernosal injection and transurethral therapy are both examples of second-line therapy. With a small needle, vasoactive substances are directly injected into the corpora cavernosa. Examples of vasoactive substances are prostaglandin E_1, papaverine and phentolamine. This method of treatment has the advantages to be predictable in what will happen when injecting, and to act rapidly. Both men or their partner can be trained to inject the penis. This method is independent of sexual arousal.

Possible side-effects are penile pain, penile fibrosis, bleeding, urethral pain or burning, hypotension, syncope, and priapism (1).

Vacuum constrictive devices

Another example of second-line therapy are the vacuum constrictive devices. They function by applying continuous negative pressure to the shaft of the penis. This negative pressure ensures that blood is drawn to the CC, by which the penile erection will occur. Erection is preserved by applying an elastic band at the base of the penis (1, 52). Despite the inexpensiveness and low drawback, this method is very mechanical with a cold penis sensation. This makes patients and partners very unsatisfied. A vacuum device is preferred for patients who do not want to use injections or inflatable penile prosthesis implantation (IPP). Side-effects of this therapeutic device are petechiae, penile numbness, and delayed ejaculation (1, 21).

1.6.3 Third-line therapy

Patients who do not respond to the first- and second-line treatments of ED, are called refractory ED patients. Refractory ED patients are often assigned to more invasive options of third-line therapy, such as IPP. Five important subgroups of refractory ED are: radical prostatectomy, diabetes, hypogonadal disease, vascular dysfunctions, and Peyronie's disease.

Inflatable penile prosthesis implantation

If first-line therapy did not work and second-line treatment is not effective or not preferred by the patients, third-line therapy is available (53). The third-line therapy IPP for refractory ED patients is an invasive procedure. The implantation consists of two penile cylinders with a scrotal pump. This scrotal pump is necessary for inflation, to bring fluid from the retropubic reservoir into the cylinders, which will create a penile erection (1). This surgical technique has a high satisfaction rate in both the patients (92-100%) and their partners (91-95%) (21). The major advantage of IPP is that it results in a firm penis and a simple manual use. The main complication of IPP is infection, which occurs in less than 1-3% of the patients (21, 54).

To conclude, good therapies for ED are available. However, still 40% of ED patients do not respond to first-line therapy and are assigned to much more invasive options, such as IPP. For patients who do not respond to non-invasive methods, new therapeutic targets must bring opportunities for treatment.

1.7 Research for new therapeutic targets

Most studies investigating the transcriptomic alterations in ED were mainly focused on the known candidate genes (i.e. *eNOS*) which are involved in the NO-sGC-cGMP pathway, known to contribute to ED (32, 55). But, up until now, research did not address the entire transcriptome (32). Therefore, transcriptome analysis by NGS in CC tissue of ED patients compared to non-ED controls is highly required in order to identify the genetic alterations within ED. Dysregulated genes involved in biological pathways will be evaluated, aiming to identify new potential non-invasive therapeutic targets for refractory ED patients. This is important, because current non-invasive treatment is not effective for 40% of ED patients. An important part of these refractory patients, designed to the

invasive IPP treatment, are the five subgroups mentioned before (i.e. radical prostatectomy, diabetes, hypogonadal disease, vascular dysfunctions, and Peyronie's disease). Research should therefore focus on these five specific causes of ED.

1.7.1 Potential candidate targets

The neurotransmitter H_2S is known for its relaxation capacity independent of endothelial cells, which is favourable for patients with ED. This makes H_2S pathway interesting for new therapeutic targets (12). As mentioned before, ROS could also play a role in penile erection. For this reason, enhancing antioxidants or limiting the activity of ROS-generating enzymes could be a possible therapeutically path to use in ED patients (16, 18)

Finding these new candidate therapeutic targets could give us new opportunities to help these refractory patients in a non-invasive way. For some of these refractory patients (i.e. diabetes), it is important that new targets work in a NO-independent way, such as for example the stimulation of sGC via direct stimulation resulting in NO-independent cGMP accumulation and smooth muscle cell relaxation (55). Nevertheless, by focussing on specific targets narrows the view for new specific targets are missed. This is why it is important to investigate the entire transcriptome with NGS.

1.8 Research setup

Refractory ED patients can typically be divided in five subgroups. However, little is known about these non-responders. It is still not understood why they do not respond to the present medical aid. To overcome this high medical need for these refractory ED patients, research must focus on searching for new potential therapeutic targets. Before transcriptomic analysis can show alterations in gene expression, RNA extraction of CC must be optimised and proven to be of high quality for reliable RNA sequencing (RNAseq) and real time quantitative polymerase chain reaction (qPCR) experiments. However, little is known about RNA extraction from CC. We **hypothesized** that high quality RNA can still be isolated from biobank-stored CC samples and be used for downstream applications and further analysis of the pathophysiological pathways involved in ED. For this hypothesis, there were three main **objectives**:

1. to optimise RNA extraction from CC of refractory ED patients;
2. to optimise cDNA synthesis from this CC RNA for real time qPCR;
3. to optimise library preparation from this CC RNA for RNAseq.

The first objective aimed to optimise the RNA extraction from biobank-stored CC samples from refractory ED patients, because little is known about extraction of RNA from this type of tissue. Within the second objective, we aimed to produce cDNA samples with good quality to perform a quality check qPCR. To conclude, in our third objective, we aimed to deliver RNA samples with sufficient quantity and quality that could be used for a successful library preparation for RNAseq.

2 Materials and methods

2.1 Experimental design

The University Biobank Limburg (UBiLim) has an ongoing collection of CC tissues, starting in 2010. Two hundred and eighty-eight CC samples have been collected by Prof. Dr. Van Renterghem to date (06/06/2019), which are stored in RNA*later* or are fresh frozen. This study was approved by the Ethical Committee of the Jessa Hospital and Hasselt University (Hasselt, Belgium) (10.57/uro10.04). After written informed consent, CC tissue from ED patients was obtained after IPP implantation at the Jessa Hospital (Hasselt, Belgium). CC Samples were transported on ice from the Salvator campus to the Virga Jesse campus of the Jessa Hospital. The time needed between collection and processing of the CC samples was called the time until processing.

This used collection consisted of CC tissue from refractory ED patients, who did not respond to PDE5-I. If desired, these patients were offered different types of therapy, such as intracavernosal injections. Patients who had androgen blockade therapy were excluded for this study. Five subgroups of refractory ED were investigated: radical prostatectomy, diabetes, hypogonadal disease, vascular dysfunctions, and Peyronie's disease.

After the CC tissue was collected from ED patients or healthy control patients, it was immediately placed in a tube with RNA*later*. The sample was stored in RNA*later* overnight at 4°C. After this, the sample was dried and put into liquid nitrogen for long-term storage. Snap frozen tissue was collected on a cloth with natrium chloride (NaCl). Immediately after collecting, the sample was put on ice and transported to the biobank where they emerged the sample in liquid nitrogen. Healthy control tissue was obtained from organ donation patients at the Jessa Hospital, more precisely from heart beating, brain-dead donors who started the process for organ donation. After written informed consent was received from a legal guardian, the CC tissue was removed from the penis of the deceased person. Because of the small number of male organ donors each year (approximately six), a collaboration with the research group of prof. Javier Romero-Otero in Madrid (University hospital Ramón y Cajal, Spain) was established to receive CC tissue samples from 20 healthy persons undergoing organ donation. Ethical approval was obtained by the Spanish research institute and the ethical committee of Jessa Hospital. Samples were stored in liquid nitrogen.

2.2 Sample size

Sample size calculation for RNA-sequencing was based on Ching *et al.* (56). The sample size was estimated at six per group with a statistical power greater than 80%. Five refractory ED groups (vascular dysfunction, diabetes, radical prostatectomy, Peyronie's disease and hypogonadism) will be compared to one healthy control group. For RNA-sequencing, we aim at analysing 6 samples per group (6 groups) based on the article of Ching *et al*. However, for the performed pilot, only 3 samples of 2 groups (vascular dysfunction and diabetes) were analysed.

2.3 RNA extraction

Different RNA extraction techniques were used and compared. An overview of the different tissue collection methods, homogenisation methods, lysis buffers and RNA extraction methods were shown in **Table 1**.

Table 1: Overview of the tested RNA extraction methods. *This table gives an overview of all methods of RNA extraction that were tested. Different tissue collection methods, homogenisation methods and lysis buffers were compared to optimise quantity and quality of RNA.*

Tissue collection method	**Tissue homogenisation**	**Tissue lysis buffer**	**RNA extraction method**
RNA*later* Snap frozen	GentleMACS dissociator	RLT	RNeasy mini kit
RNA*later*	Pestle	RLT	RNeasy mini kit
RNA*later* Snap frozen	Pestle	QIAzol	RNeasy mini kit
RNA*later*	Glass beads	RLT	QIAcube with RNeasy mini kit
RNA*later* Snap frozen	Pestle	QIAzol	QIAzol method – precipitation
RNA*later*	GentleMACS dissociator	RLT	RNeasy mini kit with proteinase K

2.3.1 RNeasy mini kit

Two collection methods were tested, RNA*later* and snap frozen. Between 10 and 26mg of CC tissue was used for the RNeasy mini kit. In addition, also two different homogenisation methods were tested, the mechanical GentleMACS (Miltenyi Biotec GmbH, Bergisch Glabach, Germany, n = 7 with 4 samples RNA*later* and 3 samples snap frozen) vs. the manual pestle (n=2, RNA*later*). When using the GentleMACS, CC tissue was placed in a M-tube with RLT buffer (Qiagen). For this mechanic homogenisation, the automatic RNA 02_01 modus with a rotation speed up to 4000 rounds per minute (rpm) during 85 seconds was used on the GentleMACS. Two lysis buffers were tested. In one test, the usual RLT buffer was used, while in a second these RLT buffer was replaced by the QIAzol buffer. The manufacture's protocol was followed in both cases, with only a change in lysis buffer.

RNA was manually extracted from the CC tissue using the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA was eluted in 30µl volume.

2.3.2 RNeasy mini kit with additional proteinase K step

Five CC samples collected with RNA*later* were tested in this method in combination with the GentleMACS dissociator. The used automatic protocol for the GentleMACS was described before. Around 20mg of tissue was used to test this method. The RNeasy mini kit protocol was used with an additional proteinase K (Qiagen) step after homogenizing the tissue to mimic the working of the RNeasy fibrous tissue mini kit (Qiagen). Next, the manufacture's protocol of RNeasy mini kit was followed as usual. Thirty µl of RNA was eluted.

2.3.3 QIAcube robot

This method only used RNA*later* tissue as collection method. Three samples were analysed. The input on the QIAcube robot was around 20mg of CC tissue. Glass beads (Sigma-Aldrich, Darmstadt, Germany) were used to homogenize CC tissue according to a standard protocol used at KULeuven. Glass beads were used in an automized mechanical way.

The QIAcube (Qiagen) is an automatic extraction robot, using the programmed protocol for RNeasy mini kit. After this extraction, 30µl of RNA was eluted.

2.3.4 QIAzol method

Four CC samples collected in RNA*later* and five CC samples collected snap frozen were tested. Different weights of CC tissue were tested, ranging from five to 89 mg. Homogenisation of the tissue was done in a manual way, using a pestle.

The QIAzol method, based on precipitation of RNA, was tested. QIAzol was added to the CC tissue and homogenised with the pestle. The sample was centrifuged at 12,000g for 10 minutes at 2-8°C. The cleared homogenate was transferred into a new 1.5ml Eppendorf tube and the samples were incubated five minutes at room temperature (RT). Chloroform was added and the samples were shaken vigorously by hand and incubated at RT for three minutes. The samples were centrifuged at 12,000g for 15 minutes at 2-8°C. The upper, aqueous phase was carefully transferred to a new 1.5ml Eppendorf tube without disturbing the interphase. By adding isopropanol, the RNA was precipitated. The samples were mixed by inverting several times and incubated at RT for 10 minutes. The precipitated RNA was collected by centrifugation at 12,000g for 10 minutes at 2-8°C. The RNA pellet was washed with 75% ethanol and air-dried for 10 minutes at RT. Samples were mixed by vortexing and centrifuged at 7500g for 5 minutes at 2-8°C. The RNA was dissolved in 20µl pre-heated RNAse-free water (40°C) and the solution was incubated for 10 minutes at 55°C before RNA quantity and quality was checked.

2.3.5 Quantity and quality analyses of extracted RNA

The quantity and purity of the extracted RNA was determined by NanoDrop-1000 UV-Vis spectrometer (Thermo Scientific, Wilmington, DE, USA). The RNA quantity and integrity were assessed by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, United States) using the 6000 Nano kit (Agilent), according to the instructions provided by the manufacturer. A marker was accompanied with each RNA sample, to bracket the overall sizing range, aligning ladder data with sample data. Via the electropherogram, the RNA quality and integrity were

determined. For each sample, an RNA Integrity Number (RIN) score was obtained. The extracted RNA was stored at -80°C.

2.4 Real time quantitative polymerase chain reaction

2.4.1 cDNA quality control for quantitative polymerase chain reaction

Total RNA was transcribed into cDNA with SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, Massachusetts, USA), which was performed according to the manufacturer's instructions.

Duplex qPCR for housekeeping genes hydroxymethylbilane synthase (*HMBS*) and haemoglobin subunit beta (*HBB*) was performed on a Rotor-Gene Q (Qiagen) running an in-house developed qPCR (57, 58). The *HMBS* gene was a short fragment (47bp), while *HBB* was a long fragment (268bp), measuring quantity and quality, respectively. The master mix consisted of Absolute qPCR SYBR green mix (AbGene, Portsmouth, NH), *HMBS* and *HBB* primers (Eurogentec, Seraing, Belgium) and DNase/RNase-free water. Total reaction volume was 20µl. SuperScript™ IV First-Strand Synthesis System recommended that cDNA was 10% of the total volume, resulting in 2µl of cDNA input. The input concentration was 96ng of cDNA. The final concentration of 0.2µM for each *HMBS* primer and 0.3µM for each *HBB* primer. The used positive control was cDNA obtained from RNA from white blood cells of patients with acute myeloid leukaemia (AML). A non-template control (NTC) was also present in the reaction. All reactions were performed in duplicate.

qPCR was performed to assess quality and quantity of reverse transcribed RNA. The sequences of the used primers for qPCR were summarized in **Table 2**.

Table 2: Primers used for real-time qPCR quality control of cDNA *Within this table, the primers are depicted that were used to perform RT-qPCR used for quality control of cDNA. Primer sequence (5'-3') of both primers are shown in table 1. HMBS, hydroxymethylbilane synthase; HBB, haemoglobin subunit beta; TMf, forward primer; TMr, reverse primer*

Gene name (human)	Primer sequence 5'…3'	Melting temperature	Amplicon size
HMBS-sh-TMf	TTCCAGGGATTTGCCTCAC	58°C	47 bp
HMBS-sh-TMr	GAGGCAAGGCAGTCATCA	56°C	47 bp
HBB-TMf	GAAGAGCCAAGGACAGGTAC	62°C	268 bp
HBB-TMr	CAACTTCATCCACGTTCACC	60°C	268 bp

The in-house developed qPCR repeated the following cycle 40 times: 95°C for five seconds, 60°C for ten seconds and 72°C for 20 seconds.

Results of the duplex qPCR were described as quality control (QC) values for *HMBS-HBB* primer mix. Cycle threshold (Ct) data normalization was done to the positive control. The QC values (also called delta Ct (ΔCt)) were calculated relative to the positive control (white blood cells from bone marrow), as follows: Ct sample—Ct positive control. The mean Ct values for reversed transcribed RNA from CC ranged between 20.23 and 21.73. The mean Ct value for the positive control amounted to 23.06. The melting curves were analysed to assess the specificity and peak height for each amplicon.

2.5 Transcriptomic analysis

Six samples were sent to GenomicsCore (Leuven, Belgium). Three samples were obtained from vascular dysfunction patients and three samples were obtained from diabetes patients. RNA was extracted with the RNeasy mini kit (Qiagen) according to the manufacturer's instructions, after CC tissue homogenisation with the GentleMACS dissociator (Miltenyi). Transcriptomic analysis was performed using Quantseq (Lexogen, Vienna, Austria) by GenomicsCore. Before library preparation, the RNA samples were purified with MinElute purification kit (Qiagen), according to manufacturer's protocol. Up to 17µl of RNA was purified per sample using the MinElute purification kit according to manufacturer's protocol. A final quality control was performed using the standardized protocols of Lexogen.

After library preparation, Quantseq analysis was performed on these six samples using the Illumina HiSeq 4000NGS (Illumina, San Diego, CA, USA). After alignment of the raw RNA-sequencing data to the reference genome (GRCh38.p12) using Hisat2 version 2.1.0, counts were normalized and differential gene expression was performed using the DESeq2 (59).

2.6 Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) 25.0 (SPSS IBM, Chicago, IL, USA). Values are expressed as means ± standard error or median with interquartile range (IQR). Normality was assessed by the Shapiro-Wilk test. When normality is assumed, Leven's test was assessed to check equality of variance. Comparison between two groups were analysed with an independent samples T-test. When data were not normally distributed, a non-parametric Mann-Whitney U was used. Correlation was checked with the Pearson correlation test if normality was assumed. A p-value below 0.05 was considered statistically significant. For differential gene expression, the adjusted p-value (p_{adj}) was used. P_{adj} was used for multiple testing with the Benjamini-Hochberg procedure, which controls false discovery rate.

3 Results

3.1 RNA extraction optimisation

The RNA extraction of CC was optimised during this research thesis. Several homogenisation and RNA extraction methods were compared in order to determine which method resulted in the best RNA quantity and quality for future downstream analysis, such as RNAseq experiments. Generally, for high-quality and integer RNA, the RIN value is preferable above six, and both the optimal optical density (OD)260/280 and OD 260/230 ratios need to range between 1.8 and 2.2. The minimal OD260/280 and OD260/230 value for RNAseq is 1.5.

3.1.1 GentleMACS dissociator and RNeasy mini kit with

The CC tissue samples from ED patients was collected in RNAlater solution or snap frozen. The effect of these two different collection methods on the RNA extraction was studied. The CC tissue, in RNAlater or snap frozen, was mechanically homogenised with the GentleMACS dissociator. Subsequently, RNA was extracted using the RNeasy mini kit. For RNA*later* four CC samples were extracted, compared to three CC samples collected snap frozen.

With the GentleMACS dissociator, a very good homogenisation of the CC tissue samples was achieved, since there were no leftovers of the tissue visible. Independent of collection method, the sample was completely dissolved in the RLT lysis buffer as depicted in **Figure 3**.

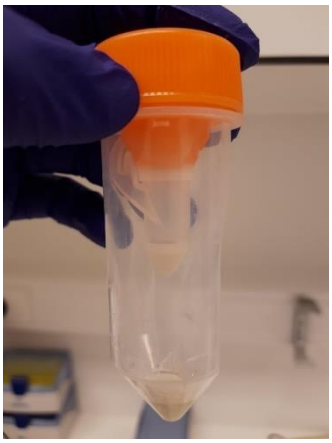


Figure 3 *Homogenisation of corpus cavernosum samples with the GentleMACS without any leftovers. The tissue is completely dissolved.*

As shown in **Table 3** the RNA concentrations varied a lot between the samples. Generally, the highest RNA concentration was received from CC tissue samples with a weight of 13,6 and 17,3 mg, independent of collection method. Samples that weighed more than 19,0 mg or less than 12,4 mg had a lower concentration compared to samples that weighed between 13,6 and 17,3 mg.
First of all, the concentration in RNA*later* samples was amounted to 21.69 ± 9.02 ng/µl measured with NanoDrop and 13.0 ± 5.79 ng/µl assessed with the Bioanalyzer. For the snap frozen samples, the concentration measured with the NanoDrop was 13.39 ± 2.42 ng/µl and with the Bioanalyzer 13.5 ± 3.01 ng/µl.

The OD260/280 value felt within the optimal range of 1.8 to 2.2 in six out of seven times in both collection methods. One RNA*later* sample did not fit the range. For the RNA*later* samples, the mean OD260/280 amounted to 2.16 ± 0.08. Only one value was higher than 2.2, and did not meet the required range. Within the snap frozen group, the mean OD260/280 value was 2.07 ± 0.07, with all samples fitting in the desired range. The OD260/230 values were all below 1.8. Within the RNA*later* group, the mean 260/230 value was 0.33 ± 0.16. Compared to the snap frozen group, this was more or less the same with a mean OD260/230 of 0.31 ± 0.14.

All samples were analysed using an electrophoretic assay. Results were shown as gel-like images (bands) and electropherograms (peaks). A marker was used to bracket the overall sizing range,

aligning ladder data with sample data. RIN values were based on the electrophoretic trace of the sample. The ideal range for RIN samples was between six and ten. RIN scores higher than seven indicated high-quality RNA. When the RIN was lower than 7, partial or complete RNA degradation could occur. For two RNA*later* samples, the RIN values were determined (N/A) by the Bioanalyzer. The obtained RIN values were higher than six, except for one snap frozen sample (18CC010).

Generally, the RIN values in the RNA*later* group were higher than in the snap frozen group. The mean RIN of RNA*later* samples was 8.3 ± 0.6 compared to 6.4 ± 0.85 for the snap frozen samples. Moreover, the RIN of snap frozen samples taken in 2015 (15CC008) was higher than the RIN of samples taken in 2017 and 2018 (17CC033 and 18CC010).

There were no significant differences in concentration, OD value or RIN between RNA*later* and snap frozen samples.

Table 3: Results RNA optimisation with GentleMACS, RLT buffer and RNeasy mini kit. *CC samples were homogenised with the GentleMACS and RNA was extracted with the RNeasy mini kit and. Within this optimisation, RLT buffer was used. For each sample, the used collection method, RNAlater or snap frozen, was shown. Furthermore, the concentration (ng/µl) measured on the NanoDrop as well as on the Bioanalyzer was presented. Both OD260/280 and OD260/230 were obtained by the NanoDrop. Lastly, RIN was measured with the Bioanalyzer. RNA concentrations varied between the samples. The obtained OD260/280 value fitted the range. OD260/230 was lower than expected and did not fit the range. RIN values that were determined were in four out of five times higher than six. CC, corpus cavernosum; OD, optical density; RIN, RNA integrity number.*

Sample number	**Collection method**	**Concentration NanoDrop (ng/µl)**	**OD260/280 (1.8-2.2)**	**OD260/230 (1.8-2.2)**	**RIN**	**Concentration Bioanalyzer (ng/µl)**
17CC033	RNA*later*	34.95	2.23	0.72	8.9	14.0
18CC010	RNA*later*	9.41	2.08	0.12	N/A	4.0
18CC007	RNA*later*	3.19	2.35	0.03	N/A	5.0
15CC007	RNA*later*	39.2	1.99	0.45	7.7	29.0
17CC033	Snap frozen	8.57	1.93	0.11	6.1	11.0
15CC008	Snap frozen	16.2	2.15	0.57	8	10.0
18CC010	Snap frozen	15.39	2.14	0.25	5.1	19.50

The electropherograms obtained by the Bioanalyzer were shown in **Figure 4**. Figure 4A was an example of an electropherogram obtained by high-quality RNA. The first peak was the marker followed by two distinct peaks at the 18S fragment (second peak) and the 28S fragment (third peak), both representing the ribosomal subunits. This electropherogram showed intact, high-quality RNA. The corresponding RIN value was 8.9. In contrast in Figure 4B the RNA is partially degraded. There were still two peaks visible in the 18S fragment and 28S fragment, but less pronounced. The matching RIN value was 5.1, which did not fit the range. Lastly, figure 4C showed no peak in the 18S fragment nor in the 28S fragment. There was no RNA measured, resulting in a flat electropherogram

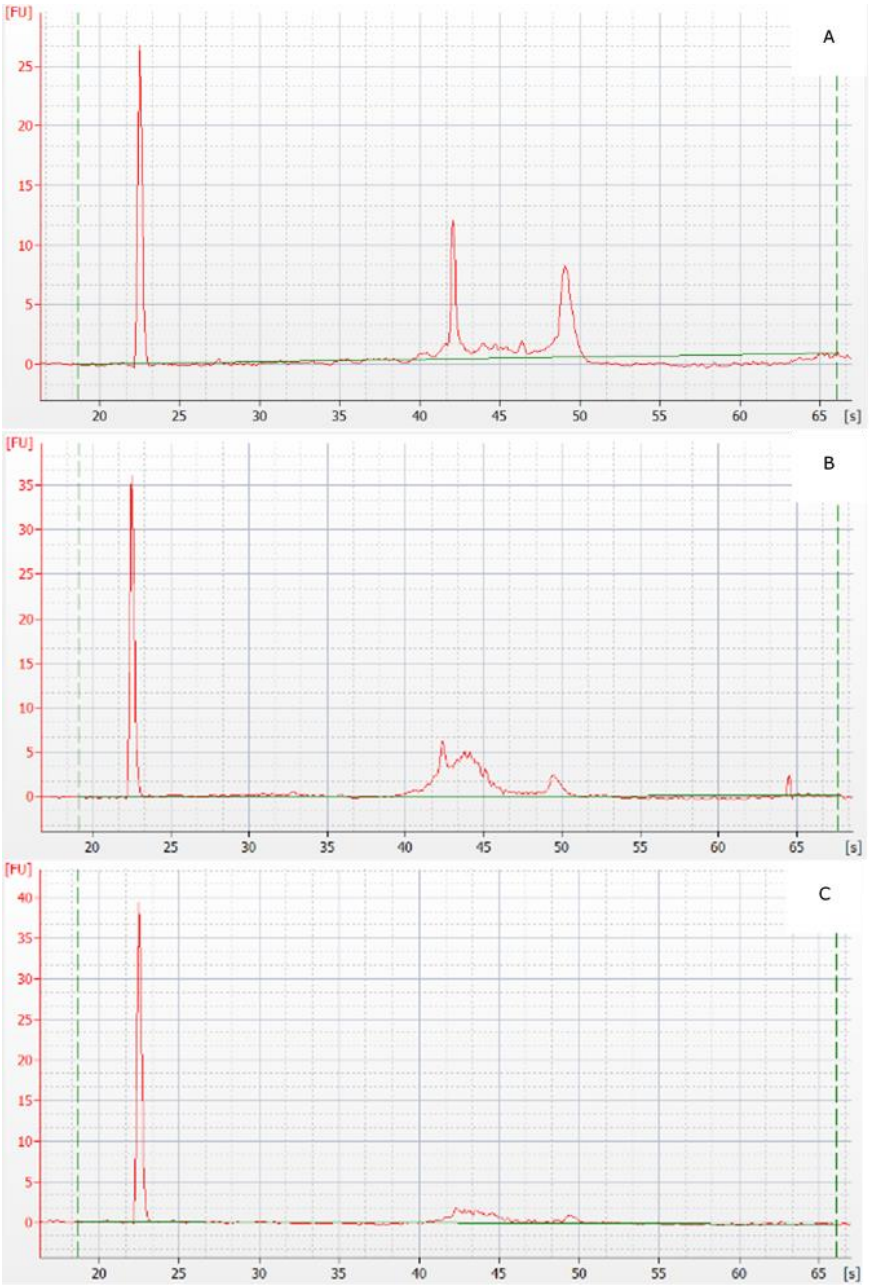


Figure 4: Electropherograms of RNA samples.
A. The first peak represented the marker, the second and third peak represented 18S and 28S fragment. The RIN value was 8.9. This was an example of high quality, non-degraded RNA.
B. The first peak represented the marker, the second and third peak represented 18S and 28S fragment with a baseline noise in between. The RIN was 5.1, which resembles degraded RNA.
C. Only one peak was visible, which was the marker. No peak was visible in the 18S and 28S region, which represented as an electropherogram with no RNA present. No RIN value was calculated.

3.1.2 Pestle and RNeasy mini kit with RLT buffer

In order to test and compare different homogenisation methods, the GentleMACS dissociator was replaced by manual method, using a pestle. For the RNA extraction, the RNeasy mini kit was used according to manufacturer's protocol. Here, the CC tissue was collected in RNA*later*. In this setting, only two samples were studied.

Manual homogenisation of CC with a pestle was labour-intensive. The tissue was not fully homogenized and did not dissolve in the RLT lysis buffer. Leftovers of tissue were visible in the RLT buffer after homogenisation.

As depicted in **Table 4**, the concentrations measured with NanoDrop were low, with a mean of 13.10 ± 5.87 ng/µl. The concentrations measured with the Bioanalyzer were comparable to those measured with NanoDrop, amounting to 13.0 ± 1.41. The mean OD260/280 value was 2.18 ± 0.23. However, only one of the two OD260/280 values fit in the range of 1.8-2.2. Furthermore, the OD260/230 values were very low, with a mean of 0.40 ± 0.09. In addition, only one RIN value was above six.

Table 4: Results optimisation using a pestle in combination with the RNeasy mini kit and RLT buffer. *CC was homogenised using a manual method, the pestle. Only one collection method was used, RNAlater. Furthermore, the table shows the concentration (ng/µl) measured on the NanoDrop as well as on the Bioanalyzer. OD260/280 as well as the OD260/230 were obtained by the NanoDrop. In conclusion, the RIN value was measured with the Bioanalyzer. The Bioanalyzer gave different RNA concentrations compared to the NanoDrop. OD260/280 fitted the range in one out of two samples. OD260/230 was below 1.8. One RIN value was above six, while the other did not meet this range. CC, corpus cavernosum; OD, optical density; RIN, RNA integrity number*

Sample number	Collection method	Concentration NanoDrop (ng/µl)	OD260/280 (1.8-2.2)	OD260/230 (1.8-2.2)	RIN	Concentration Bioanalyzer (ng/µl)
18CC021	RNA*later*	17.25	2.02	0.46	5.1	14.0
18CC022	RNA*later*	8.95	2.35	0.33	7.9	12.0

3.1.3 Pestle and RNeasy mini kit with QIAzol buffer

As third method, a manual pestle homogenisation followed by an RNA extraction with RNeasy mini kit was performed. Instead of the standard RLT lysis buffer of the RNeasy mini kit, the QIAzol lysis buffer was used. In total, three RNA*later* as well as three snap frozen CC tissue samples were used.

The manual homogenisation with a pestle did not completely dissociate nor dissolve the CC sample, as demonstrated with the arrow in **Figure 5**. Clear leftovers of the tissue were still visible after homogenisation.

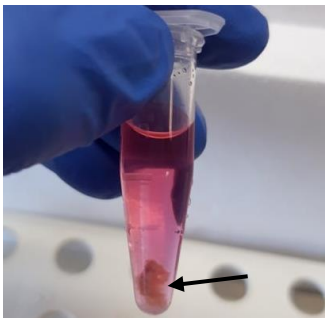


Figure 5: Leftovers of corpus cavernosum tissue after homogenisation with a pestle in QIAzol buffe. *Using a manual homogenisation such as a pestle, did not resolve the tissue. Leftovers were still visible, pointed to by the arrow.*

Table 5 showed the results of the combination of the use of a pestle and RNeasy mini kit with the QIAzol buffer instead of the RLT buffer. For the RNA*later* samples, the RNA concentration was 31.87± 12.73 ng/µL measured with the NanoDrop compared to a concentration of 17.67 ± 10.60 ng/µl on the Bioanalyzer. The mean OD260/280 in these samples was 1.75 ± 0.22. The OD260/230 was below 1.8 with a mean of 0.68 ± 0.59. The average RIN value in the RNA*later* samples was 7.42 ± 0.98. The mean concentration of snap frozen CC samples was 19.28 ± 7.87 and 13.67 ± 8.01 ng/µl, measured on NanoDrop and Bioanalyzer, respectively. Additionally, they did not meet 1.8 for the OD260/280 and OD260/230, with a mean of 1.73 ± 0.13 and 0.41 ± 0.25 respectively. The RIN value of snap frozen samples exceeded 7, with a mean RIN of 7.78 ± 0.32.

Again, there were no significant differences in concentration, OD value or RIN between RNA*later* and snap frozen samples.

Table 5: Results optimisation of the pestle with RNeasy mini kit and QIAzol buffer. *A manual homogenisation method was used to dissociate the tissue. RNA was extracted using the RNeasy mini kit with QIAzol lysis buffer instead of RLT lysis buffer. Two collection methods were tested, three RNAlater samples and three snap frozen samples. During this optimisation, concentrations (ng/µl) were measured on NanoDrop and Bioanalyzer. Both OD260/280 and OD260/230 were obtained by the NanoDrop. The Bioanalyzer also calculated the RIN value. Concentrations did vary between NanoDrop measurements and Bioanalyzer calculations. OD260/280 was below two and OD260/230 varied a lot, with no value meeting the 1.8. One value was not determined by the Bioanalyzer, while the other values all meet the threshold value of six. CC, corpus cavernosum; OD, optical density; RIN, RNA integrity number.*

Sample number	Collection method	Concentration NanoDrop (ng/µl)	OD260/280 (1.8-2.2)	OD260/230 (1.8-2.2)	RIN	Concentration Bioanalyzer (ng/µl)
18CC027	RNA*later*	26.5	1.94	1.29	6.3	29.0
18CC022	RNA*later*	22.7	1.79	0.65	7.8	16.0
18CC021	RNA*later*	46.4	1.51	0.11	8.15	8.0
18CC027	Snap frozen	23.6	1.86	0.67	7.6	21.5
18CC022	Snap frozen	24.05	1.74	0.41	8.0	14.0
18CC021	Snap frozen	10.2	1.60	0.17	N/A	5.5

3.1.4 Glass beads with the QIAcube robot

The automatic QIAcube robot was tested. Homogenisation of the CC tissue was performed by glass beads. This robot extracted RNA from CC tissue according to the programmed RNeasy mini kit protocol. Only three RNA*later* samples were used in this method.

The glass beads were not a good method to dissociate the CC tissue. Leftovers of the CC tissue were still present in the tube after homogenisation.

The concentrations measured by NanoDrop were all below 5ng/µl as shown in **Table 6**. Furthermore, the OD260/280 and OD260/230 values were extremely low, not reaching 0.1. In comparison the manual RNA extraction using the RNeasy mini kit, the robot gave no better results. RIN and Bioanalyzer concentrations were not measured due because of low RNA quantity and quality.

Table 6: Results optimisation of glass beads in combination with the QIAcube robot. *CC was homogenised using glass beads. The QIAcube robot extracted RNA according to the RNeasy mini protocol. With this protocol, only three RNAlater samples were tested. The concentration (ng/µl), OD260/280 and OD260/230 were measured with NanoDrop. Concentrations were all below 5ng/µl. Furthermore, the OD260/280 and OD260/230 values did not meet 0.1. CC, corpus cavernosum; OD, optical density; RIN, RNA integrity number*

Sample number	Collection method	Concentration (ng/µl)	OD260/280 (1.8-2.2)	OD260/230 (1.8-2.2)
18CC027	RNA*later*	<5	<0.1	<0.1
18CC016	RNA*later*	<5	<0.1	<0.1
17CC004	RNA*later*	<5	<0.1	<0.1

3.1.5 Pestle and QIAzol method

To test a manual method without the use of columns, the QIAzol method was chosen.

For the homogenisation of the tissue, a pestle was used. However, as shown in **Figure 6** there were still leftovers of the CC tissue present in the tube (indicated by the arrows). The tissue is not completely dissolved in the QIAzol lysis buffer.

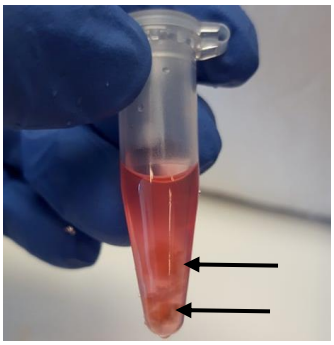


Figure 6: Leftovers of corpus cavernosum tissue after homogenisation with a pestle in QIAzol buffer. *The arrows show the leftovers of CC tissue in the QIAzol buffer. The manual pestle homogenisation did not resolve the tissue*

Table 7 showed the results of this method. Two collection methods were compared within this method, namely four RNA*later* and five snap frozen CC samples. For the RNA*later* samples, the average concentration measured with NanoDrop was 127.38 ± 37.96 ng/µl. These samples were also measured on the Bioanalyzer, resulting in a mean concentration of 50.5 ± 37.56 ng/µl. For the snap frozen samples, the obtained mean concentration on NanoDrop amounted to 156.88 ± 43.57 ng/µl, while on the Bioanalyzer the RNA concentrations were lower with a mean amount of 72.5 ±17.26 ng/µl. In general, there was no direct link between the weight of the tissue compared to the concentration when using the QIAzol method. Even when a low amount of tissue (5.1mg) was used, there is still a good yield in RNA.

The OD260/280 value of the RNA*later* samples was 1.68 ± 0.07. The RNA*later* samples gave a mean OD260/230 of 0.48 ± 0.17. For the snap frozen samples, the OD260/280 and OD260/230 value amounted on average to 1.8 ± 0.03 and 1.01 ± 0.25, respectively.

The RIN value of the RNA*later* samples amounted to 5.77 ± 1.93. This was higher than the mean RIN values of the snap frozen samples, which were on average 5.28 ± 0.60.

There were no significant differences between the two collection methods regarding concentration, OD values and RIN value.

Table 7: Results optimisation using a pestle with the QIAzol method – precipitation. *CC was homogenised in a manual way, using the pestle. Four RNAlater samples and four snap frozen samples were used. Within this optimisation, a precipitation method was assessed, namely the QIAzol method. RNA concentration (ng/µl) was measured with the NanoDrop and Bioanalyzer. Purity was assessed with the OD260/280 and OD260/230 value. The RIN value, calculated with the Bioanalyzer, suggested the integrity of the extracted RNA. Concentrations measured with NanoDrop were higher than concentrations measured with the Bioanalyzer. Four out of nine OD260/280 were above 1.8. All OD260/230 values were lower than 1.8. Determined RIN values varied a lot, with four out of seven determined RIN values above six. CC, corpus cavernosum; OD, optical density; RIN, RNA integrity number*

Sample number	Collection method	Concentration NanoDrop (ng/µl)	OD260/280 (1.8-2.2)	OD260/230 (1.8-2.2)	RIN	Concentration Bioanalyzer (ng/µl)
17CC007	RNA*later*	167.2	1.83	0.98	7.6	163.0
18CC024	RNA*later*	66.3	1.54	0.22	N/A	7.5
18CC025	RNA*later*	61.35	1.61	0.37	7.8	18.0
18CC026	RNA*later*	214.65	1.75	0.33	1.9	13.5
18CC026	Snap frozen	285.85	1.83	1.56	6.6	61.0
17CC007	Snap frozen	60.8	1.78	0.70	4.2	51.0
18CC027	Snap frozen	137.8	1.80	0.21	4.3	63.0
18CC025	Snap frozen	73.65	1.71	1.4	6.0	47.0
18CC026	Snap frozen	226.3	1.87	1.18	N/A	140.5

3.1.6 GentleMACS dissociator and RNeasy mini kit with RLT buffer and additional proteinase K

In order to mimic the procedures of the RNeasy fibrous tissue mini kit (Qiagen), the RNeasy mini kit was used with an additional proteinase K step. Homogenisation was performed mechanically with the GentleMACS dissociator. The CC tissue was completely dissolved in the RLT buffer and no leftovers were visible. All studied samples (five) were collected in RNA*later*. **Table 8** showed the measured values for this method. The mean concentration measured on the NanoDrop was 9.09 ± 2.20 ng/µl. This is slightly higher than the RNA concentration determined with the Bioanalyzer, which was 7.8 ± 1.85 ng/µl. The OD260/280 fitted the desired range, with a mean of 1.93 ± 0.11. OD260/230 was below 1.8, with a mean of 0.58 ± 0.18. The average RIN amounted to 7.56 ± 0.39.

Table 8: Optimisation of the GentleMACS with RNeasy mini kit with an additional proteinase K step. *During this optimisation, the GentleMACS was used as homogenisation method. Five RNAlater samples were selected. The standard RNeasy mini protocol was used as described before, but after homogenisation an additional proteinase K step was added. Concentration (ng/µl) was measured using NanoDrop and Bioanalyzer. NanoDrop also measured the OD260/280 and OD260/230. RIN values were calculated with the Bioanalyzer. Concentrations were rather low and differ slightly from the concentrations measured with Bioanalyzer. One out of the five OD260/280 values did not meet the range. All OD260/230 values were below 1.8. The RIN values were very good, al overreaching six. CC, corpus cavernosum; OD, optical density; RIN, RNA integrity number*

Sample number	Collection method	Concentration NanoDrop (ng/µl)	OD260/280 (1.8-2.2)	OD260/230 (1.8-2.2)	RIN	Concentration Bioanalyzer (ng/µl)
18CC004	RNA*later*	4.97	2.05	0.34	7.0	3.5
18CC012	RNA*later*	7.99	1.87	0.58	6.7	4.0
18CC015	RNA*later*	4.56	1.52	0.05	7.3	7.5
18CC044	RNA*later*	11.66	2.16	0.8	8.4	12.0
19CC009	RNA*later*	16.26	2.07	1.11	8.6	12.0

Conclusively, RNA was ideally collected in RNA*later*. The weight used was ideal not above 20mg. The best method to homogenize tissue was in a mechanical way using the GentleMACS dissociator. RNeasy mini kit was the outstanding way to extract RNA from CC tissue. When using this RNeasy mini kit, the RLT buffer was the leading lysis buffer to use.

3.2 Study population

To perform the final RNA extraction, the optimal method was chosen, being homogenisation with GentleMACS dissociator and subsequent RNA extraction with the standard protocol (using RLT buffer) of the RNeasy mini kit. Furthermore, to start, the RNA*later* CC tissues were preferred. RNA extracted from 29 CC samples were extracted using this method. However, two samples were excluded because of incorrect and unreliable OD260/280 measurements (OD260/280 ≥ 10).

In addition, an extra statistical analysis was performed for the 'time until processing' to see if this was a confounder which needed to be accounted for. This was defined as the time between collection and processing of the CC samples. Shapiro-Wilk test did not assumed normality. Median values with IQR were calculated. The median time until processing was 74.0 (59-129) minutes. There was no significant correlation between time until processing and the concentration, OD260/280 or OD260/230.

From the 29 extracted RNA samples, six RNA samples were used for RNAseq and three RNA samples were used for qPCR. The remaining samples will be used in future RNAseq and qPCR experiments.

3.3 Duplex qPCR with *HBB* and *HMBS*

After cDNA synthesis, duplex qPCR for the *HMBS* and *HBB genes* was performed with three samples in order to determine the quality and quantity of the reverse-transcribed RNA. The mean QC values were depicted in **Table 9**. The ΔCt values or QC values, were negative, as shown in **Figure 7**.

Table 9: Mean Ct values and ΔCt values calculated for the *HBB* and *HMBS* genes. *The mean QC values of the three samples and the positive control were shown. RNA extracted from CC was transcribed into cDNA. Duplex qPCR was performed with HMBS and HBB genes. With the mean Ct values, the QC values were calculated as follows:* $Ct_{sample} - Ct_{positive\ control}$*. Ct, threshold cycle; CC, corpus cavernosum; qPCR, quantitative polymerase chain reaction; HMBS, hydroxymethylbilane synthase; HBB haemoglobin subunit beta;* QC value; Quality control value *; AML, Acute myeloid leukaemia*

Sample number	Disease	Mean Ct value	QC value
18CC012	Diabetes	20.18 ± 0.07	-2.88 ± 0.07
18CC028	Diabetes	21.66 ± 0.09	-1.40 ± 0.09
18CC037	Vascular	21.58 ± 0.11	-1.48 ±0.11
Positive control	AML	23.06 ± 0.00	/

Both *HBB* and *HMBS* fragments were formed in this duplex qPCR. This was analysed in the raw melting curve data, as depicted in **Figure 8**. The first peak (around 73°C) in the melting curve represented the formed primer-dimers. The short *HMBS* fragment rose at a temperature of 79°C. The *HBB* fragment peaked around 86°C. The red curve was the positive control (AML). Two reverse-transcribed RNA samples (orange and green) had a shifted peak at 86.5°C shifting away from the positive control, as demonstrated by the arrows in **Figure 8**. The orange melting curve had a melting temperature of 86°C. The green curve peaked lower compared to the positive control, but had a melting temperature of 86.5°C.

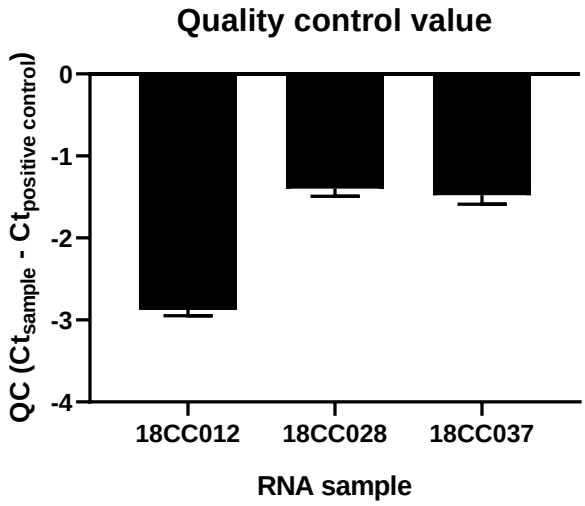


Figure 7: Qualitative and quantitative control of RNA. *RNA from CC samples was transcribed into cDNA. After cDNA synthesis, a duplex qPCR was performed with the genes HMBS and HBB. Three different RNA samples from CC were analysed. The QC values were all below zero. RNA, ribonucleic acid; CC, corpus cavernosum; HMBS, hydroxymethylbilane synthase; HBB haemoglobin subunit beta; qPCR, quantitative polymerase chain reaction; mean ± standard deviation*

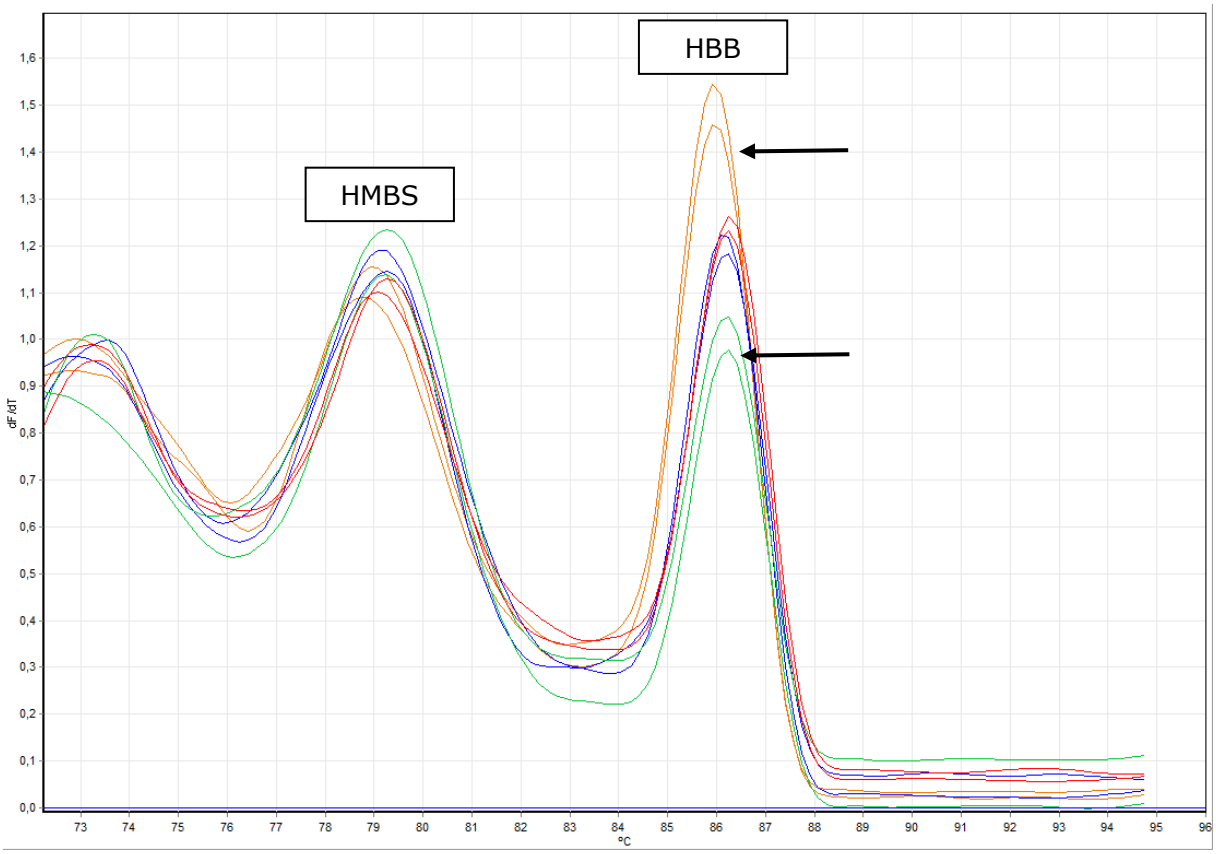


Figure 8: Melting curves analysis of duplex qPCR with HMBS and HBB genes. *Melting curves were generated after a duplex qPCR with HMBS and HBB genes. The first peak were primer dimers around 73°C, followed by the second peak around 79°C represented the HMBS gene. The third peak around 86.5°C resulted in the HBB gene. The red curve presented the positive control. The arrows indicated a slightly change in temperature (°C) in the melting curves (green and orange melting curve). HMBS, hydroxymethylbilane synthase; HBB haemoglobin subunit beta; qPCR, quantitative polymerase chain reaction.*

3.4 Transcriptomic analysis

For transcriptomic analysis, the Quantseq method (Lexogen) was chosen. Six samples were sent to GenomicsCore (Leuven, Belgium), which included three RNA samples of vascular dysfunction patients and three RNA samples of diabetic patients. All CC samples were collected in RNA*later*. They were homogenized using the GentleMACS dissociator and RNA extraction was performed with the RNeasy mini kit.

Within the vascular patients' group, the mean concentration measured with the NanoDrop was 21.26 ± 3.37 ng/µl. The concentration on the Bioanalyzer, 11.83 ± 6.84 ng/µl, slightly differed from the concentration measured with NanoDrop. The average OD260/280 did not fit the normal range, with a value of 2.24 ± 0.07. The OD260/230 was lower than the required 1.8, namely 0.47 ± 0.15. The RIN value was calculated at 8.6 ± 0.15.

On the NanoDrop, the average concentration of RNA isolated from CC of the diabetic group was 15.7 ± 2.83 ng/µl. This was higher than the concentration measured with the Bioanalyzer, which was 8.83 ± 1.59 ng/µl. The average value of OD260/280 was 2.21 ± 0.14. The OD260/230 amounted to 0.50 ± 0.26. To conclude, the RIN outreached the six, with a mean of 8.03 ± 0.41.

3.4.1 Purification and library preparation

Before the library preparation could be performed, GenomicsCore performed a purification of the RNA samples with the MinElute purification kit (Qiagen) to increase the sample quality. Although RNA yield was decreased after purification, the total amount of RNA was high enough (threshold of 100ng required for RNAseq). Moreover, OD260/230 values of four out of six RNA samples rose above 1.5, as referred to in yellow in **Table 10**. OD260/280 values were not changed during the MinElute purification and therefore not depicted in the table.

Table 10: Concentration and OD260/230 values of the RNA samples after MinElute purification. *Before using the RNA samples of a library preparation for Quantseq analysis, the samples needed to be purified. Before purification, the OD260/230 values were lower than 1.5. After using the MinElute purification kit, the values raised and in four out of six samples, the values increased above the critical 1.5 value. OD, optical density*

		Before MinElute Purification			After MinElute Purification		
Sample number	**Disease**	**Concentration NanoDrop (ng/µl)**	**RNA yield (ng) in 30µl**	**OD260/230 (1.8-2.2)**	**Concentration NanoDrop (ng/µl)**	**RNA yield (ng) in 17µl**	**OD260/230 (1.8-2.2)**
18CC018	Vascular	21.39	641.7	0.12	26.1	443.7	1.95
19CC005	Vascular	27.04	811.2	0.58	19.9	338.3	1.94
18CC038	Vascular	15.36	460.8	0.55	11.8	200.6	1.64
18CC017	Diabetes	20.24	607.2	1.02	14.5	246.5	1.74
17CC007	Diabetes	10.51	315.3	0.24	13.6	231.2	0.88
18CC043	Diabetes	16.35	490.5	0.23	14.4	244.8	1.19

3.4.2 Differential gene expression with Quantseq analysis

After a successful library preparation and quality control, GenomicsCore performed a Quantseq analysis. Fifty genes were differential expressed between both test groups (**Appendix Table 1**). From these fifty differentially expressed genes, only three genes were statistically different between the vascular group and the diabetes group, namely, Angiopoietin-like 4 (*ANGPTL4,* p_{adj} *= 0.002),*

Ribosomal protein L9 (*RPL9,* p_{adj} *= 0.002*) and KAT8 Regulatory NSL complex Subunit 1-Antisense RNA 1 (*KANSL1-AS1,* p_{adj} *= 0.04*). As shown in **Figure 9**. The three genes *ANGPTL4* (*p = 0.04*), *RPL9* (*p = 0.01*) and *KANSL1-AS1* (*p = 0.02*) were significantly higher expressed in the diabetes group, compared to the vascular patient group.

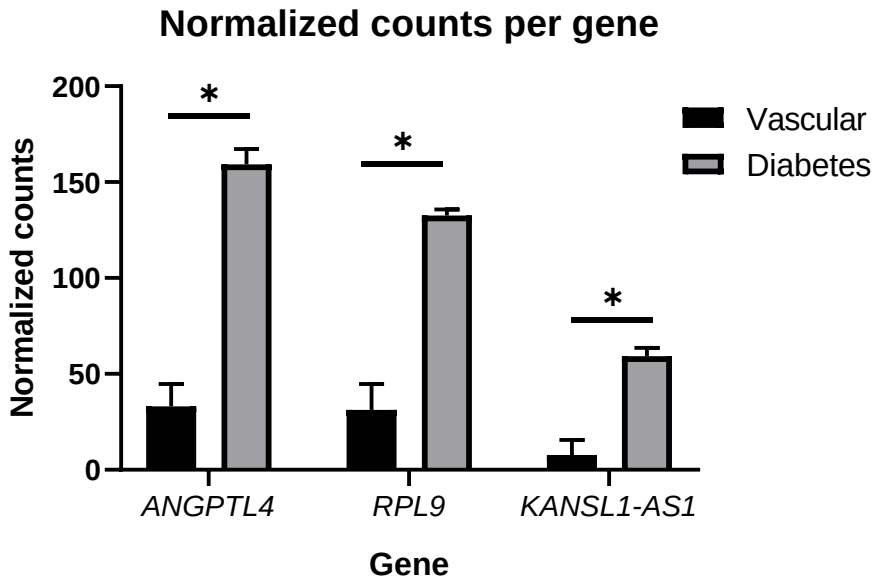


Figure 9: Difference in gene expression with Quantseq based on normalized counts with DEseq2 *(59). After normalization of the counts per gene, the median with interquartile range per group was calculated. The significant difference in gene expression between the vascular group and the diabetic group was determined using a T-test analysis. Each group was based on three samples. All genes were significantly more expressed in the diabetes group compared to the vascular group, ANGPTL4 (p = 0.04), RPL9 (p = 0.01) and KANSL1-AS1 (p = 0.02).*
ANGPTL4, Angiopoietin-like4; RPL9, Ribosomal protein L9; KANSL1-AS1; KAT8 Regulatory NSL complex Subunit 1-Antisense RNA 1. Median with interquartile range

4 Discussion

The condition ED is explained as the inability to attain or maintain a penile erection sufficient for sexual intercourse (1). There are many reasons why men develop ED, this may be due to relationship problems or lifestyle habits (i.e. smoking, no exercise) (2). Nonetheless, there are also patients who suffer from a serious medical condition (i.e. diabetes, prostate cancer treatment,…).

To get an erection, the NO-pathway must be activated, resulting in a decrease in Ca^{2+} and relaxation of the smooth muscle cells in the CC tissue of the penis. Therefore, the key player in receiving an erection is the CC tissue. Up until now, research mainly focussed on the known NO-pathway. Current medical aids, such as Viagra®, are also mostly based on this pathway. However, this medication offers no relief to 40% of ED patients (1). Patients who do not respond to medical offers are called refractory ED patients. These refractory ED patients can be divided in five subgroups, namely patients suffering from vascular dysfunction, diabetes, Peyronie's disease, hypogonadism and total prostatectomy. These refractory patients are assigned to more invasive options, such as IPP. Therefore, the ultimate goal is to find new potential therapeutic targets, which do not depend on NO production (55). These new potential therapeutic targets could be beneficial for refractory ED patients, resulting in non-invasive treatment options.

This CC is of great importance for penile erection, because it is expandable tissue allowing blood flow to enter the penis. This results in penile erection. The CC tissue was described as fibroelastic tissue (8).

With the ultimate goal of transcriptomic analysis with NGS (i.e. Quantseq), high-quality and quantitively RNA should be obtained from CC tissues. However, few researches have been done on transcriptomic alternations in CC. Therefore, hardly any publications on RNA extraction from this CC tissue could be found. For this reason, RNA extraction from CC tissue first had to be optimised.

Our biobank contains CC tissue from 288 patients. These samples were collected in RNA*later* or snap frozen. The CC tissues are stored in the biobank since 2010. The snap frozen samples that were collected in 2015 have a better RIN value compared to the snap frozen samples of 2017 and 2018. Over the years, the surgeries of Dr. Van Renterghem moved to another campus, increasing the time until processing. In 2015 the IPP operations were done in the Jessa hospital, campus Virga Jesse. The CC tissue was immediately transferred to the laboratory of UBiLim (Jessa hospital, campus Virga Jesse) and processed. Nowadays, the IPP operations are performed on the Salvator campus of the Jessa hospital. Therefore, the CC tissue samples need to be transported from Salvator campus to Virga Jesse campus, which takes about one to two hours to get the samples to the laboratory for processing. The time needed to transfer the samples from the operating room to the laboratory for processing and storage is longer now compared to 2015. Consequently, the time up until processing increased. Previous research on other types of tissue (i.e. esophageal adenocarcinoma) confirms this observed effect of time on the quality of the RNA samples (60). With our small sample size, no significant correlation was found between the time until processing and the quality of RNA. However, larger studies must be performed in order to confirm this statement. Nonetheless, the delay between sampling and fresh-freezing must be minimized as much as possible.

Furthermore, our results suggested higher mean RIN values in RNA*later* samples, however no significant difference was found between RNA*later* and snap frozen tissue. RNA*later* samples were immediately emerged into the RNA*later* solution and transported on ice, whereas the snap frozen samples were placed on a clot with NaCl and transported on ice. This difference in tissue collection can have an effect on the RIN value of the RNA samples. Since the CC tissue samples are stored on ice for at least one hour, cold ischemia may be induced, which could possibly lead to RNA degradation (61). In case of a longer transport from operating room to biobank before processing, RNA*later* is recommended for the protection of the quality of RNA (62).

Another important part to take into account is the amount of tissue that will be used for RNA extraction. Our results recommended an amount of 13 to 18mg as ideal weight, preferably not exceeding the 22mg of tissue for the RNeasy mini kit. As for the QIAzol method, a maximum weight up to 100mg can be used according to in-house protocols. With the RNeasy mini kit, it is important not to use to much CC tissue, because it is a column-based approach. The maximum weight is preferably between 15-20mg according to the manufacture's protocol (63). This maximum weight applies for both the manual use of the RNeasy mini kit as well as the use in a QIAcube robot. When too much starting material is loaded, the column will clog and RNA will not bind anymore (63). However, no clear link was established between the weight and the obtained concentration in the QIAzol method.

The best way to homogenize the CC tissue is with a mechanical dissociator such as the GentleMACS dissociator. With this method, the samples were completely dissolved and homogenized in the lysis buffer. When compared to the manual homogenisation using a pestle, the tissue does not dissolve completely. There were still leftovers visible in the lysis buffer. To efficiently extract all RNA, it is required to dissolve the CC tissue completely, since incomplete homogenization results in inefficient binding of RNA to the RNeasy mini column (63). The GentleMACS is more powerful than the pestle, resulting in fully homogenised CC tissue. Research showed that a mechanical homogenisation is better than a manual homogenisation (62).

When comparing the RNeasy mini kit with the standard RLT lysis buffer and the RNeasy mini kit with QIAzol lysis buffer, no major differences were viewed in concentration, OD-values or RIN values. RLT is a lysis buffer for non-fatty tissue, while QIAzol is a lysis buffer used for lysis of fatty and normal tissue before RNA isolation. In our opinion, both lysis buffers are usable for RNA extraction from CC.

Generally, there is a difference between the measurements of concentration with the NanoDrop and the measurements with the Bioanalyzer. NanoDrop measures all the nucleotides (i.e. DNA leftovers) present in the extraction product, while the Bioanalyzer focusses specific on RNA nucleotides only. The Bioanalyzer is more reliable in measuring RNA concentrations (62). Overall, the total RNA yield was higher than 100ng. For Quantseq, the required threshold value for total RNA is 100ng (64). When compared to the RNeasy mini kit, the highest total RNA concentrations were measured with the QIAzol method. The QIAzol method is based on precipitation, and precipitates all nucleotides, while in the RNeasy mini kit only RNA binds to the column. With the QIAzol method, leftovers of DNA can be present in the precipitated solution, resulting in higher concentrations measured with

NanoDrop. After measuring concentration of the QIAzol method RNA samples with the Bioanalyzer, the concentrations were lower compared to concentration measured with NanoDrop. To remove any DNA present in the RNA samples, an additional DNase digestion step should be performed (63, 65). However, the effect of this extra digestion step has not been tested in this study. Another possible explanation for the observed difference in measured concentrations is that the spin columns of the RNeasy mini kit only binds RNA molecules longer than 200 bases, which selectively excludes most of the ribosomal- and transport-RNA (63). Since precipitation does not have this selection, it precipitates all RNA molecules, which may raise the RNA concentration.

When comparing the yield obtained by the QIAcube, which is the automatic version of the RNeasy mini kit, to the manual RNeasy mini kit, concentrations were below 5 ng/µl using the QIAcube. This is lower than the concentration obtained with the manual method, concluding that the manual RNA extraction with the RNeasy mini kit is a better solution for CC tissue. This is not what should be expected, because the robot is a closed system, avoiding contamination. Furthermore, the QIAcube robot is an automatic device, which should result in more accurate pipetting. However, note that the homogenisation between the manual and automatic RNeasy mini kit was different. This may have an influence on these results. Both homogenisation methods were mechanical, but the manual method uses the GentleMACS, while the automatic QIAcube used glass beads. This may have an effect, because the beads did not completely homogenise the tissue, leaving leftovers, which may result in a lower RNA concentration.

Purity of the RNA samples is determined by the OD260/280, which is measured spectrophotometrically, and should be in the range of 1.8-2.2. The 260nm wavelength determines the presence of DNA and/or RNA in the sample, because the ultraviolet light will be absorbed by the DNA and/or RNA. Proteins absorb ultraviolet light at 280nm. The higher the contamination with proteins, the lower the OD260/280 value will be. The OD260/280 values are in the optimal range in the RNeasy mini kit method. The RNeasy mini kit only binds RNA on the column, leading to less protein contamination in the RNA sample. Within the QIAzol method, the OD260/280 values were lower because the extraction does not only consist of RNA, but also proteins.

For the OD260/230, the 230nm wavelength measures the presence of salt, carbohydrates, peptides and aromatic compounds, and should be in the range of 1.8-2.2. The OD260/230 value is extremely low for all methods. This may be due to organic contaminations. The RNeasy mini kit contains RLT buffer, which has a high concentration of guanidine isothiocyanate. Organic contamination causes low OD260/230 values if residues stay present. Furthermore, β-mercaptoethanol is added to the RLT buffer, which can also affect the OD260/230 value (63, 66). A 260/230 value under 1,5 cannot be used for RNAseq, due to interference of organic components present in the RNA extraction. Quantseq is less susceptible for this contamination, but only on condition that the OD260/280 is between 1.9 and 2.1; and the RIN value is above six (64, 67).

RIN stands for RNA integrity number. A marker is used to bracket the overall sizing range, aligning ladder data with sample data. Via the electropherogram, the RNA quality and integrity were determined. A RIN above six is known to be intact RNA, whereas a RIN lower than six indicates

degraded RNA. Overall, the best RIN values are obtained with a combination of the GentleMACS homogenisation and RNeasy mini kit, starting with RNA*later* CC tissue samples. Due to a low total RNA concentration, the RIN value could not be determined (N/A) in some samples. Another problem with measurement of the RIN value was an unexpected signal in the 5S region. Additional peaks in the 5S region are often seen when a phenol or QIAzol method is used due to small RNA contamination. They can be removed by using Qiagen columns (i.e. RNeasy mini kit), which will remove small RNA's (63). Samples with a RIN value above eight are optimal for RNAseq experiments, the minimum RIN value is six. When the RIN value is below six, the samples are too degraded to use for RNAseq experiments (68, 69).

In conclusion, RNA*later* is preferably used for CC tissues. Furthermore, a mechanical homogenisation is best for this type of tissue. The RNeasy mini kit with RLT buffer is favourable method to extract RNA from CC tissue. The combination of these optimisation results will be used in following RNA extractions of CC for future downstream analysis such as qPCR and RNAseq.

With the ultimate goal to perform a qPCR for validation of the differentially expressed genes in a larger cohort study, cDNA must be produced with our RNA samples and the respective quality of RNA. To assess whether our RNA had a good quality to perform a qPCR, a duplex qPCR was carried out. Duplex qPCR of reverse-transcribed RNA from CC samples was performed using both *HBB* and *HMBS*. The *HMBS* gene is a short fragment of 47 bp and represents the quantity of cDNA that was produced from RNA from CC. The *HBB* gene is a long fragment of 268 bp and reveals the quality of cDNA. Resulting from the duplex qPCR, the QC values were lower than zero, resulting in both good quality and quantity RNA. A good QC value is related with a low QC value (57). Using a duplex qPCR, the raw melting curves are of great importance. Melting curve analysis determines the presence of primer-dimers and ensures reaction specificity. Primer-dimers were present in the melting curves, decreasing PCR efficiency and obscures analysis (78). Weak interactions can occur between primers, often after 30 cycles, which will result in binding to each other instead of the target (78, 79). Complementary of just one nucleotide can cause primer-dimers. Careful primer-design is important for reducing primer-dimer formation (79). Furthermore, SYBR green binds to non-specific double stranded DNA. As primer-dimers are double stranded, SYBR green will bind and give a fluorescent signal. A probe based qPCR is more specific and will not bind to primer-dimers, eliminating them from the qPCR results (80). The melting curve showed that both gene fragments were amplified, which can prove that the QC values were obtained from both the *HBB* and *HMBS* amplicon. However, the melting curves were not perfect in comparison with the used positive control. For the *HBB* gene, the curves moved to a different temperature compared to the positive control. Different reasons can possibly explain this. To begin, the cDNA synthesis of the positive control was performed in another run then the three samples. Minor differences in the procedure could result in the small differences in the obtained cDNA samples and consequently possibly also in the melting curves. In addition, the positive control was obtained from a completely different disease, AML and from a different kind of tissue, namely blood. Furthermore, the *HBB* gene has several splicing variants. Differences in splicing variants can result in different amplicon lengths with different melting temperatures. To confirm that the obtained melting curves originate from the *HBB* gene fragment, a gel electrophoreses or

sequencing analysis must be performed. As long as the peaks do not correspond entirely with the positive control, there can be no direct conclusion about the detection of the targeted fragment. In future experiments, specificity can be added by using probe-based qPCR (i.e. TaqMan probe). The SYBR green qPCR does not ensure whether the amplification reaction consists of target, non-target or a mixture of both (78). However, within our objective it was mainly aimed to prove that cDNA synthesis of our RNA samples is possible. Therefore, we can conclude that our RNA seems of sufficient quality and quantity for successful cDNA preparations and downstream qPCR analysis. In future research, the obtained potential therapeutic targets will be validated with a qPCR in a larger cohort study.

Quantseq analysis is a sequencing method that sequences libraries close to the 3' end of polyadenylated RNAs. Oligo dT primers will bind at the polyadenylated site of the mRNA. Only one fragment per transcript is generated, so this can be directly linked to the number of reads mapping to a gene to its expression. Whit classic whole RNAseq, mRNA is first fragmented and random primers will be used for strand synthesis. Multiple fragments per transcript are generated. The advantage of Quantseq over RNAseq is that fewer reads are necessary reducing the time for sequencing and the price for sequencing. Quantseq is also less sensitive for RNA impurity. Whole transcriptome RNAseq, however, detects 15% more differentially expressed genes compared to Quantseq (67, 70). Differential gene expression analysis is important to determine new potential therapeutic targets for patients with refractory ED. Quantseq analysis of six samples: three vascular samples and three diabetic samples, showed a significant difference gene expression in three genes. These genes were more expressed in the diabetic group than in the vascular group. The expression of *ANGPTL4* is upregulated in diabetic patients with ED compared to vascular patients with ED. *ANGPTL4* is known for its role in cancer, lipoprotein metabolism and glucagon-to-insulin regulation (71-73) and is mostly expressed in the liver. The link with insulin metabolism may explain the increase in gene expression in the diabetic group compared to the vascular group. *ANGPTL4* is also known for wound heling in diabetic mice. Chong *et al.* showed that *ANGPTL4* stimulated the STAT3-mediated NO production with angiogenesis and wound healing as a result (74). As NO production in CC is important (1, 19), this gene may play a role in the regulation of this NO-production, because an increase in gene expression was observed in diabetic patients. However, this gene has never been addressed before in literature of (refractory) ED. Therefore, further research and pathway analysis is highly required to further establish the possible role of this gene. *RPL9* is a ribosomal protein, mostly expressed in ovary or bone marrow. It is also known to be highly expressed in colorectal carcinoma (75). Ribosome-related genes are associated with vasculitis (76). As vasculitis is a risk factor for ED (1, 21), the link between ED and *RPL9* can possibly be found within this field of research. However, again, there is still no knowledge in literature about its actions and role within CC or ED, nor with diabetes. Further analysis is required. A third gene being significantly different expressed was *KANSL1-AS1*. No knowledge is present in literature about the specific function of the gene. This gene is known to be expressed in fat and spleen, but about its function not much is known (77). Conclusively, also further research must be performed. Comparison with healthy controls will deliver

more information about the exact role of these genes in diabetes or ED and whether or not these genes are also expressed in healthy controls.

In future research, 36 samples will be analysed. Five subgroups of refractory ED will be compared with a healthy control group. It is expected that there will be differential gene expression between the five subgroups and the healthy controls. Furthermore, differences in gene expression between groups will also be expected, due to the different pathophysiology's of the underlying diseases. In addition, to be sure that one of this change in gene expression should be linked to ED and not to diabetes, the comparison with these healthy controls will be very important. Conclusively, due to the small sample size and the low number of genes significantly differentially expressed, pathway analysis was not possible. In the future, when more samples are analysed, if more genes are differentially expressed, pathways analysis will be performed to find new treatment options for these patient groups with refractory ED.

5 Conclusion and synthesis

Up to 40% of all ED patients do not respond to first-line treatment, such as Viagra®. They are often destined to more invasive options, such as IPP. Five subgroups of patients with refractory ED are men with diabetes, vascular dysfunctions, Peyronie's disease, hypogonadism or a total prostatectomy. These patients need non-invasive options, resulting in research searching for new potential therapeutic targets. To find these therapeutic targets, CC was used to extract RNA. In our biobank, CC is stored since 2010. However, little is known in literature about the ideal method to extract RNA from CC. Conclusively, optimisation of the RNA extraction was performed. RNA quantity and quality were determined.

Within this research thesis, our results proved that downstream application and further analysis of the pathophysiological pathways involved in ED is possible with biobank-stored RNA*later* CC tissue samples. CC tissue was ideally collected in RNA*later* to stabilize RNA. The homogenisation of CC tissue was best using a mechanical homogenisation method, such as the GentleMACS. The best method to extract RNA was with RNeasy mini kit in combination with the RLT buffer. For RNAseq, samples need to be purified using the MinElute kit. Furthermore, the obtained quality and quantity of this RNA is sufficient for qPCR analysis. Purified RNA samples can also be used in RNAseq experiments, especially in Quantseq analysis.

Future research on searching for new potential therapeutic targets in ED is highly required. After this research thesis, the RNA extraction and optimisation of CC tissue from refractory ED patients is established. To be sure the time until processing does not influence the quantity and quality of RNA, more samples must be compared in the future. In addition, a larger RNAseq experiment must be conducted with the five known refractory groups compared to healthy controls, because new therapeutic targets are necessary to treat these patients in a non-invasive way. Based on power analysis, this research requires six samples in each group with a total of 36 samples. This larger group of samples will enable us to get a deeper and broader view of the differential genes expressed and to couple this to a pathway analysis. With pathway analysis, the exact role the genes within a pathway will be clarified. Furthermore, RNA samples with a high RIN value (RIN >8) and a OD260/280 and OD260/230 value above 1.5 can be used for whole transcriptome analysis, which detects 15% more genes compared to the used Quantseq analysis (81). After RNAseq analysis, the differentially expressed genes must be validated in a larger patient cohort with qPCR. To improve the specificity of the qPCR, a probe-based qPCR must be designed for the desired genes. In the future, refractory ED patients may be helped in a non-invasive way using the new potential therapeutic targets.

References

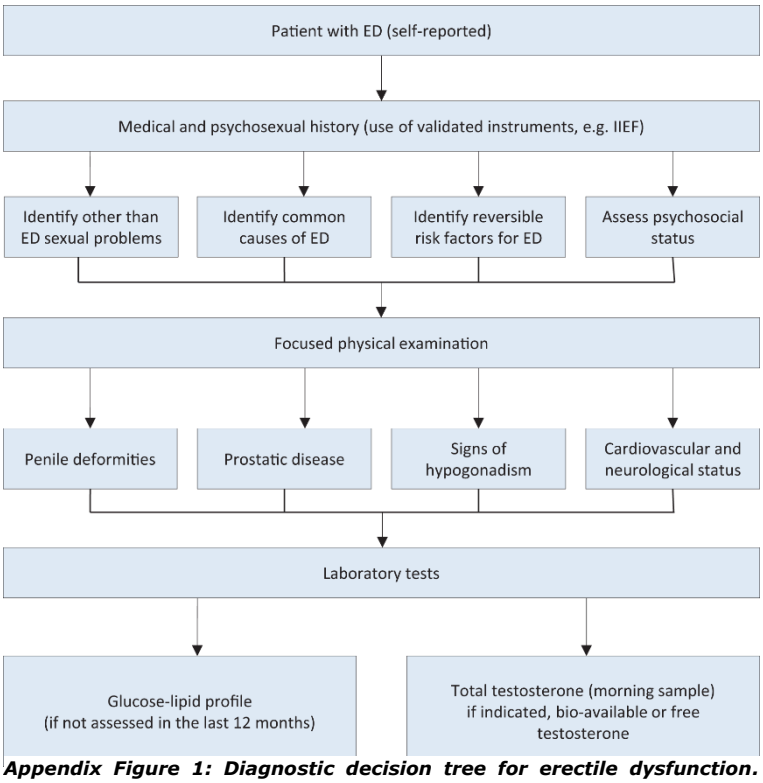
1. Shamloul R, Ghanem H. Erectile dysfunction. The Lancet. 2013;381(9861):153-65.
2. Kirby M. The circle of lifestyle and erectile dysfunction. Sexual medicine reviews. 2015;3(3):169-82.
3. McCabe MP, Althof SE. A systematic review of the psychosocial outcomes associated with erectile dysfunction: Does the impact of erectile dysfunction extend beyond a man's inability to have sex? The journal of sexual medicine. 2014;11(2):347-63.
4. Chambers SK, Chung E, Wittert G, Hyde MK. Erectile dysfunction, masculinity, and psychosocial outcomes: a review of the experiences of men after prostate cancer treatment. Translational andrology and urology. 2017;6(1):60.
5. Feldman HA, Goldstein I, Hatzichristou DG, Krane RJ, McKinlay JB. Impotence and its medical and psychosocial correlates: results of the Massachusetts Male Aging Study. The Journal of urology. 1994;151(1):54-61.
6. Braun M, Wassmer G, Klotz T, Reifenrath B, Mathers M, Engelmann U. Epidemiology of erectile dysfunction: results of the 'Cologne Male Survey'. International journal of impotence research. 2000;12(6):305.
7. Simopoulos EF, Trinidad AC. Male erectile dysfunction: integrating psychopharmacology and psychotherapy. General hospital psychiatry. 2013;35(1):33-8.
8. Unknown. PHYSIOLOGY OF ERECTION AND PATHOPHYSIOLOGY OF ERECTILE DYSFUNCTION. In: Publishers NS, editor. International Book of Erectile Dysfunction. New York: Nova Science Publishers; 2016.
9. Mas M. Molecular mechanisms of penile erection. Arch Esp Urol. 2010;63(8):589-98.
10. Wespes E. Erectile dysfunction: the medical treatment. European Urology Supplements. 2013;12(2):13-7.
11. Qiu X, Villalta J, Lin G, Lue TF. Role of hydrogen sulfide in the physiology of penile erection. Journal of andrology. 2012;33(4):529-35.
12. Bivalacqua TJ, Usta MF, Champion HC, Kadowitz PJ, Hellstrom WJ. Endothelial dysfunction in erectile dysfunction: role of the endothelium in erectile physiology and disease. Journal of Andrology. 2003;24(S6):S17-S37.
13. Dean RC, Lue TF. Physiology of penile erection and pathophysiology of erectile dysfunction. Urologic Clinics. 2005;32(4):379-95.
14. Uvin P, Albersen M, Bollen I, Falter M, Weyne E, Linsen L, et al. Additive effects of the Rho kinase inhibitor Y-27632 and vardenafil on relaxation of the corpus cavernosum tissue of patients with erectile dysfunction and clinical phosphodiesterase type 5 inhibitor failure. Bju international. 2017;119(2):325-32.
15. Sopko NA, Hannan JL, Bivalacqua TJ. Understanding and targeting the Rho kinase pathway in erectile dysfunction. Nature Reviews Urology. 2014;11(11):622.
16. Decaluwé K, Pauwels B, Boydens C, Van de Voorde J. Treatment of erectile dysfunction: new targets and strategies from recent research. Pharmacology Biochemistry and Behavior. 2014;121:146-57.
17. Kupelian V, Link CL, McKinlay JB. Association between smoking, passive smoking, and erectile dysfunction: results from the Boston Area Community Health (BACH) Survey. European urology. 2007;52(2):416-22.
18. Papaharalambus CA, Griendling KK. Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. Trends in cardiovascular medicine. 2007;17(2):48-54.
19. Yafi FA, Jenkins L, Albersen M, Corona G, Isidori AM, Goldfarb S, et al. Erectile dysfunction. Nature Reviews Disease Primers. 2016;2:16003.
20. Traish AM, Stottrup C, van Renterghem K, Achten R, Roy S. Density and distribution of connexin 43 in corpus cavernosum tissue from diabetic and hypogonadal patients with erectile dysfunction. Hormone molecular biology and clinical investigation. 2013;13(1):7-12.
21. Hatzimouratidis K. EAU guidelines on Male Sexual Dysfunction 2018.
22. Martin-Morales A, Sanchez-Cruz JJ, De Tejada IS, Rodriguez-Vela L, Jimenez-Cruz JF, Burgos-Rodriguez R. Prevalence and independent risk factors for erectile dysfunction in Spain: results of the Epidemiologia de la Disfuncion Erectil Masculina Study. The Journal of urology. 2001;166(2):569-75.
23. El-Sakka AI. Erectile dysfunction: Association of risk factors and medical comorbidities with male sexual dysfunctions. The journal of sexual medicine. 2007;4(6):1691-700.
24. Lewis RW, Fugl-Meyer KS, Corona G, Hayes RD, Laumann EO, Moreira Jr ED, et al. Definitions/epidemiology/risk factors for sexual dysfunction. The journal of sexual medicine. 2010;7(4pt2):1598-607.
25. Ponholzer A, Temml C, Mock K, Marszalek M, Obermayr R, Madersbacher S. Prevalence and risk factors for erectile dysfunction in 2869 men using a validated questionnaire. European urology. 2005;47(1):80-6.
26. Gatti A, Mandosi E, Fallarino M, Radicioni A, Morini E, Maiani F, et al. Metabolic syndrome and erectile dysfunction among obese non-diabetic subjects. Journal of endocrinological investigation. 2009;32(6):542-5.
27. Demir O, Akgul K, Akar Z, Cakmak O, Ozdemir I, Bolukbasi A, et al. Association between severity of lower urinary tract symptoms, erectile dysfunction and metabolic syndrome. The Aging Male. 2009;12(1):29-34.
28. Hirshkowitz M, Karacan I, Arcasoy MO, Acik G, Narter EM, Williams RL. Prevalence of sleep apnea in men with erectile dysfunction. Urology. 1990;36(3):232-4.

29. Bellinghieri G, Santoro D, Mallamace A, Savica V. Sexual dysfunction in chronic renal failure. Journal of nephrology. 2008;21(2):S113.
30. Huyghe E, Kamar N, Wagner F, Capietto AH, El-Kahwaji L, Muscari F, et al. Erectile dysfunction in end-stage liver disease men. The journal of sexual medicine. 2009;6(5):1395-401.
31. Köseoğlu N, Köseoğlu H, Ceylan E, Cimrin H, Özalevli S, Esen A. Erectile dysfunction prevalence and sexual function status in patients with chronic obstructive pulmonary disease. The Journal of urology. 2005;174(1):249-52.
32. Lopushnyan NA, Chitaley K. Genetics of erectile dysfunction. The Journal of urology. 2012;188(5):1676-83.
33. Hatzichristou D, Kirana P-S, Banner L, Althof SE, Lonnee-Hoffmann RA, Dennerstein L, et al. Diagnosing sexual dysfunction in men and women: sexual history taking and the role of symptom scales and questionnaires. The journal of sexual medicine. 2016;13(8):1166-82.
34. Rosen RC. The process of care model for evaluation and treatment of erectile dysfunction. International journal of impotence research. 1999;11(2):59.
35. Althof SE, Rosen RC, Perelman MA, Rubio-Aurioles E. Standard operating procedures for taking a sexual history. The journal of sexual medicine. 2013;10(1):26-35.
36. Rosen RC, Riley A, Wagner G, Osterloh IH, Kirkpatrick J, Mishra A. The international index of erectile function (IIEF): a multidimensional scale for assessment of erectile dysfunction. Urology. 1997;49(6):822-30.
37. Hackett G. The use of questionnaires to assess sexual function. Trends in Urology & Men's Health. 2017;8(1):17-20.
38. Davis-Joseph B, Tiefer L, Melman A, Berger RE. Accuracy of the initial history and physical examination to establish the etiology of erectile dysfunction. Urology. 1995;45(3):498-502.
39. Porst H, Burnett A, Brock G, Ghanem H, Giuliano F, Glina S, et al. SOP conservative (medical and mechanical) treatment of erectile dysfunction. The journal of sexual medicine. 2013;10(1):130-71.
40. Mutnuru PC, Ramanjaneyulu HK, Susarla R, Yarlagadda J, Devraj R, Palanisamy P. Pharmaco Penile Duplex Ultrasonography in the Evaluation of Erectile Dysfunction. Journal of clinical and diagnostic research: JCDR. 2017;11(1):TC07.
41. Lue TF, Hricak H, Marich K, Tanagho E. Vasculogenic impotence evaluated by high-resolution ultrasonography and pulsed Doppler spectrum analysis. Radiology. 1985;155(3):777-81.
42. Chisholm KE, McCabe MP, Wootten AC, Abbott JAM. Psychosocial interventions addressing sexual or relationship functioning in men with prostate cancer. The journal of sexual medicine. 2012;9(5):1246-60.
43. Esposito K, Giugliano F, Di Palo C, Giugliano G, Marfella R, D'andrea F, et al. Effect of lifestyle changes on erectile dysfunction in obese men: a randomized controlled trial. Jama. 2004;291(24):2978-84.
44. Maiorino MI, Bellastella G, Esposito K. Lifestyle modifications and erectile dysfunction: what can be expected? Asian journal of andrology. 2015;17(1):5.
45. Esposito K, Giugliano F, De Sio M, Carleo D, Di Palo C, D'armiento M, et al. Dietary factors in erectile dysfunction. International journal of impotence research. 2006;18(4):370.
46. Kolotkin R, Binks M, Crosby R, Østbye T, Mitchell J, Hartley G. Improvements in sexual quality of life after moderate weight loss. International journal of impotence research. 2008;20(5):487.
47. Polsky JY, Aronson KJ, Heaton JP, Adams MA. Smoking and other lifestyle factors in relation to erectile dysfunction. BJU international. 2005;96(9):1355-9.
48. Chew KK, Bremner A, Stuckey B, Earle C, Jamrozik K. ORIGINAL RESEARCH–ERECTILE DYSFUNCTION: Alcohol Consumption and Male Erectile Dysfunction: An Unfounded Reputation for Risk? The journal of sexual medicine. 2009;6(5):1386-94.
49. Arackal BS, Benegal V. Prevalence of sexual dysfunction in male subjects with alcohol dependence. Indian Journal of Psychiatry. 2007;49(2):109.
50. Corona G, Maggi M. The role of testosterone in erectile dysfunction. Nature Reviews Urology. 2010;7(1):46.
51. Rizk PJ, Kohn TP, Pastuszak AW, Khera M. Testosterone therapy improves erectile function and libido in hypogonadal men. Current opinion in urology. 2017;27(6):511-5.
52. Yuan J, Hoang A, Romero C, Lin H, Dai Y, Wang R. Vacuum therapy in erectile dysfunction—science and clinical evidence. International Journal of Impotence Research. 2010;22(4):211.
53. Antonini G, Busetto G, De Berardinis E, Giovannone R, Vicini P, Del Giudice F, et al. Minimally invasive infrapubic inflatable penile prosthesis implant for erectile dysfunction: evaluation of efficacy, satisfaction profile and complications. International journal of impotence research. 2016;28(1):4.
54. Mandava SH, Serefoglu EC, Freier MT, Wilson SK, Hellstrom WJ. Infection retardant coated inflatable penile prostheses decrease the incidence of infection: a systematic review and meta-analysis. The Journal of urology. 2012;188(5):1855-60.
55. Albersen M, Linsen L, Tinel H, Sandner P, Van Renterghem K. Synergistic effects of BAY 60-4552 and vardenafil on relaxation of corpus cavernosum tissue of patients with erectile dysfunction and clinical phosphodiesterase type 5 inhibitor failure. The journal of sexual medicine. 2013;10(5):1268-77.
56. Ching T, Huang S, Garmire LX. Power analysis and sample size estimation for RNA-Seq differential expression. Rna. 2014.
57. Froyen G, Broekmans A, Hillen F, Pat K, Achten R, Mebis J, et al. Validation and application of a custom-designed targeted next-generation sequencing panel for the diagnostic mutational profiling of solid tumors. PLoS One. 2016;11(4):e0154038.
58. Oben B, Cosemans C, Arijs I, Linsen L, Daniëls A, Declercq J, et al. Archival May-Grünwald–Giemsa-Stained Bone Marrow Smears Are an Eligible Source for Molecular DNA Research. Biopreservation and biobanking. 2018.

59. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome biology. 2014;15(12):550.
60. Stiekema J, Cats A, Boot H, Langers A, Balague Ponz O, van Velthuysen M, et al. Biobanking of fresh-frozen endoscopic biopsy specimens from esophageal adenocarcinoma. Diseases of the Esophagus. 2016;29(8):1100-6.
61. Grizzle WE, Otali D, Sexton KC, Atherton DS. Effects of cold ischemia on gene expression: a review and commentary. Biopreservation and biobanking. 2016;14(6):548-58.
62. Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Molecular aspects of medicine. 2006;27(2-3):126-39.
63. QIAGEN. RNeasy® Mini Handbook: QIAGEN; 2012.
64. Lexogen. QuantSeq 3ʻ mRNA-Seq Library Prep Kit · User Guide Vienna: Lexogen Gmbh; 2015 [cited 2019 April 25th]. Available from: https://www.lexogen.com/wp-content/uploads/2015/11/015UG009V0211_QuantSeq-Illumina.pdf.
65. Rio DC, Ares M, Hannon GJ, Nilsen TW. Removal of DNA from RNA. Cold Spring Harbor Protocols. 2010;2010(6):pdb. prot5443.
66. von Ahflen S. Effects of low A260/A230 ratios in RNA preparations on downstream applications. 2010.
67. Moll P, Ante M, Seitz A, Reda T. QuantSeq 3′ mRNA sequencing for RNA quantification. Nature methods. 2014;11(12).
68. Reiman M, Laan M, Rull K, Sõber S. Effects of RNA integrity on transcript quantification by total RNA sequencing of clinically collected human placental samples. The FASEB Journal. 2017;31(8):3298-308.
69. GenomicsCore. RNA SEQUENCING (RNA-SEQ) Leuven, Belgium2018 [cited 2019 May 16th]. Available from: https://www.genomicscore.be/services/rna-sequencing-rna-seq/.
70. Ma F, Fuqua BK, Hasin Y, Yukhtman C, Vulpe CD, Lusis AJ, et al. A comparison between whole transcript and 3′RNA sequencing methods using Kapa and Lexogen library preparation methods. BMC genomics. 2019;20(1):9.
71. Barja-Fernandez S, Moreno-Navarrete JM, Folgueira C, Xifra G, Sabater M, Castelao C, et al. Plasma ANGPTL-4 is Associated with Obesity and Glucose Tolerance: Cross-Sectional and Longitudinal Findings. Molecular nutrition & food research. 2018;62(10):1800060.
72. Hsieh H, Jou Y, Tung C, Tsai Y, Wang Y, Chi C, et al. Epigenetic silencing of the dual-role signal mediator, ANGPTL4 in tumor tissues and its overexpression in the urothelial carcinoma microenvironment. Oncogene. 2018;37(5):673.
73. Ingerslev B, Hansen JS, Hoffmann C, Clemmesen JO, Secher NH, Scheler M, et al. Angiopoietin-like protein 4 is an exercise-induced hepatokine in humans, regulated by glucagon and cAMP. Molecular metabolism. 2017;6(10):1286-95.
74. Chong HC, Chan JSK, Goh CQ, Gounko NV, Luo B, Wang X, et al. Angiopoietin-like 4 stimulates STAT3-mediated iNOS expression and enhances angiogenesis to accelerate wound healing in diabetic mice. Molecular Therapy. 2014;22(9):1593-604.
75. Baik IH, Jo G-H, Seo D, Ko MJ, Cho CH, Lee MG, et al. Knockdown of RPL9 expression inhibits colorectal carcinoma growth via the inactivation of Id-1/NF-κB signaling axis. International journal of oncology. 2016;49(5):1953-62.
76. Gan S, Ye B, Qian S, Zhang C, Mao J, Li K, et al. Immune-and Ribosome-Related Genes were Associated with Systemic Vasculitis. Scandinavian journal of immunology. 2015;81(2):96-101.
77. NCBI. KANSL1-AS1 KANSL1 antisense RNA 1 [Homo sapiens (human)]: NCBI; 2019 [Available from: https://www.ncbi.nlm.nih.gov/gene/?term=KANSL1-AS1.
78. Inc TFS. Real-time PCR handbook. Netherlands: Thermo Fisher Scientific Inc 2014. p. 73.
79. Brownie J, Shawcross S, Theaker J, Whitcombe D, Ferrie R, Newton C, et al. The elimination of primer-dimer accumulation in PCR. Nucleic acids research. 1997;25(16):3235-41.
80. Tajadini M, Panjehpour M, Javanmard SH. Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. Advanced biomedical research. 2014;3.
81. Xiong Y, Soumillon M, Wu J, Hansen J, Hu B, Van Hasselt JG, et al. A comparison of mRNA sequencing with random primed and 3′-directed libraries. Scientific reports. 2017;7(1):14626.

Appendix

Appendix Figure 1



Appendix Figure 1: Diagnostic decision tree for erectile dysfunction.

This decision tree gives an overview of the steps that are taken by doctors to diagnose erectile dysfunction (ED). It always starts with a medical and psychosexual history, using validated questionnaires. Next on, a physical examination and laboratory tests can be carried out (21).

Appendix Table 1

id	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
ANGPTL4	83.97	1.90	0.37	5.1898	2.1051e-07	0.0017407
RPL9	75.23	1.81	0.35	5.2378	1.6251e-07	0.0017407
KANSL1-AS1	30.09	2.44	0.54	4.5021	6.7294e-06	0.037097
INHBA	315.49	-1.25	0.30	-4.1104	3.9496e-05	0.1633
AC131025.8	463.66	-0.91	0.23	-3.9584	7.5442e-05	0.24953
STC1	380.95	-0.76	0.20	-3.8361	0.000125	0.34455
JAK2	187.35	-0.83	0.22	-3.7338	0.00018863	0.44565
STOX1	4.69	-5.60	1.55	-3.6117	0.00030425	0.50318
SULF1	806.97	-0.59	0.16	-3.6433	0.00026912	0.50318
THBS4	100.74	-1.51	0.42	-3.6307	0.00028266	0.50318
CTGF	682.39	-1.00	0.28	-3.5152	0.00043943	0.60348
DMD	1025.52	-0.55	0.16	-3.5153	0.00043918	0.60348
SCARNA9	69.91	-1.09	0.31	-3.475	0.00051087	0.60348
SNHG8	339.53	0.65	0.19	3.4906	0.00048191	0.60348
NEAT1	2227.49	-0.61	0.18	-3.456	0.00054832	0.60454
CHD2	852.92	-0.53	0.16	-3.3862	0.0007087	0.6186
DGKH	175.08	-0.85	0.25	-3.3579	0.0007855	0.6186
KLF3	232.45	-0.73	0.21	-3.3867	0.00070749	0.6186
NALCN	8.15	-4.57	1.36	-3.3693	0.0007536	0.6186
PTPN11	386.70	-0.62	0.18	-3.3736	0.00074201	0.6186
RARRES1	53.10	1.49	0.44	3.396	0.0006838	0.6186
IGJ	22.80	1.97	0.59	3.3413	0.00083385	0.62683
ADH1B	116.39	1.29	0.39	3.2844	0.0010219	0.73476
PCNT	94.56	-0.94	0.29	-3.2663	0.0010894	0.75072
FMO2	67.55	1.27	0.39	3.2442	0.001178	0.77926
CCDC23	83.94	1.07	0.33	3.2076	0.0013382	0.81866
COX6CP1	53.82	4.75	1.49	3.1776	0.001485	0.81866
H1F0	460.30	0.76	0.24	3.186	0.0014426	0.81866
PPP2R2C	68.88	2.87	0.90	3.178	0.0014827	0.81866
STAP2	8.21	3.15	0.99	3.2005	0.0013717	0.81866
MAP2	75.31	-1.10	0.35	-3.163	0.0015615	0.83302
CH25H	3.34	5.26	1.69	3.114	0.0018454	0.94576
IGHA1	35.30	1.92	0.62	3.0986	0.0019444	0.94576
RCAN1	198.18	-0.76	0.25	-3.1047	0.0019047	0.94576
CDH13	54.83	-1.56	0.51	-3.0866	0.0020245	0.95662
7SK	1.54	0.99	1.97	0.50435	0.61401	0.99982
A1BG-AS1	4.64	0.43	1.17	0.37053	0.71099	0.99982
A2M	2448.76	-0.05	0.15	-0.33941	0.7343	0.99982
A2M-AS1	10.85	-1.19	0.79	-1.5037	0.13266	0.99982
A4GALT	161.94	0.29	0.23	1.2581	0.20835	0.99982
AAAS	34.47	-0.40	0.42	-0.9529	0.34064	0.99982
AACS	60.83	0.27	0.34	0.80401	0.42139	0.99982
AADAT	5.25	0.74	1.07	0.69265	0.48853	0.99982
AAED1	54.55	0.46	0.37	1.23	0.2187	0.99982
AAGAB	141.83	0.27	0.26	1.0458	0.29565	0.99982
AAK1	120.10	-0.05	0.25	-0.2056	0.83711	0.99982
AAMDC	250.81	0.34	0.24	1.4054	0.15991	0.99982
AAMP	410.11	-0.02	0.19	-0.10634	0.91531	0.99982
AAR2	110.67	0.20	0.28	0.70681	0.47969	0.99982