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THE IMPORTANCE OF THYROID FUNCTION FOR FEMALE REPRODUCTION

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1. THE IMPORTANCE OF THYROID FUNCTION FOR FEMALE REPRODUCTION

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To Michelle, Melanie, Melodie

and Filip,

1.1 ABSTRACT

Background: Thyroid dysfunction is one of the most common endocrine disorder. Thyroid dysfunction affects the female reproductive system and can be manifested by menstrual irregularities, pregnancy loss and infertility. Unexplained infertility has an incidence of 10 to 15 % worldwide.

Aim: The general objective of this thesis was to explore the importance of thyroid function for reproduction

Material and method: Serum levels of thyroid stimulating hormone (TSH) were compared in three groups of women in early pregnancy, one high-risk group (n = 88), one low-risk group (n = 511) and a general screening group (n = 699). Serum levels of TSH, free thyroxine (fT4) and thyroid peroxidases antibodies (TPO Ab) in fertile women (n = 67) were compared to women with unexplained infertility (n = 147). By using immunohistochemistry, the protein staining of thyroid hormone receptors (TRα1 and TRβ1), TSH receptor (TSH R), mono carboxylate transporter-8 (MCT8), and type 2 iodothyronine deiodinases (DIO2)] in endometrial biopsies were compared between fertile women (n = 19) and women with unexplained infertility (n = 28). Thyroid related proteins in different part of Fallopian tube during the menstrual cycle in fertile women (n=13) were analyzed. Additionally, embryo development until day 6, in 38 human embryos cultured in standard media with T4 added were compared to development of 36 embryos cultured in standard media.

Results: The incidence of subclinical hypothyroidism and hypothyroidism was almost the same in all three study groups (almost 10 %). Hypothyroid women on levothyroxine (LT4) supplementation had in almost 50 % of cases an inadequate treatment. Women with unexplained infertility had significantly higher serum level of fT4, and lower protein staining of TRα1 and MCT8, in the endometrium. Supplementation of thyroid hormone in vitro culture media improved the blastocyst development. Additionally, we showed thyroid related proteins in the Fallopian tube.

Conclusion: It can be concluded that a general screening for thyroid dysfunction during early pregnancy, by use of TSH levels, is optimal. Furthermore, the imbalance in the thyroid system in women with unexplained infertility highlights the importance of thyroid hormone for female fertility. The improvement of blastocyst development by adding thyroid hormone in early embryo cultures and the presence of proteins related to thyroid in Fallopian tubes suggest involvement of thyroid hormone in early embryo development.

Keywords: TSH screening, early pregnancy, thyroid hormone, endometrium, Fallopian tube, embryo culture, unexplained infertility, MCT8, DIO2, thyroid hormone receptor, TSH receptor

LIST OF SCIENTIFIC PAPERS

I. **Frida Hosseini Akram**, Bengt Johansson, Gunnar Möllerström, Britt-Marie Landgren, Anneli Stavreus-Evers, Lottie Skjöldebrand Sparre
Incidence of subclinical hypothyroidism and hypothyroidism in early pregnancy
Journal of Women's health, 2017, 26, 1231 – 1235

II. **Frida Hosseini Akram**, Lottie Skjöldebrand-Sparre, Britt-Marie Landgren, Fatma Gulen Yaldir, Lusine Aghajanova, Kjell Wånggren, Anneli Stavreus-Evers
Thyroid hormone receptors in endometrium, Fallopian tubes and human- blastocysts in women with unexplained infertility compare to fertile controls *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

AITD	Autoimmune thyroid diseases
DIO2	type 2 iodothyronine deiodinases
GnRH	Gonadotropin releasing hormone
ICSI	intracytoplasmic sperm injection
IUGR	Intrauterine Growth Retardation
IVF	In vitro fertilization
MCT8	Mono carboxylate transporter-8
SCH	Subclinical hypothyroidism
SHBG	Sex hormone binding globulin
TH	Thyroid hormone
T4	Thyroxine
T3	Triiodothyronine
TBG	Thyroxine biding globulin
TRH	Thyroid releasing hormone
TSH	Thyroid stimulating hormone
TSH R	Thyroid stimulating hormone receptor
TH R	Thyroid hormone receptor
TPO-Ab	Thyroid peroxidase antibody
TRAb	Thyroid stimulating hormone receptor antibody

2. INTRODUCTION

2.1 THYROID

2.1.1 Thyroid hormones

Thyroid hormones (TH), thyroxine (T4) and 3,3', 5-trijod-L-tyronin (T3), are secreted from and stored in the thyroid gland. Thyroid hormones regulate energy homeostasis, cell proliferation, and carbohydrate-, fat- and protein metabolism.

2.1.2 The hypothalamic and the pituitary regulation of thyroid hormone secretion

The production of THs is mainly regulated by hypothalamic – pituitary – thyroid axis [1]. Thyroid stimulating hormone (TSH) stimulates the production of TH in response to thyroid releasing hormone, produced by the hypothalamus. Thyroid releasing hormone (TRH) is transported to the pituitary via the hypothalamic hypophyseal portal system. TSH and TRH are regulated by negative feedback by T3 and T4. Furthermore, thyroid hormone levels are under influence of other hormones such as glucocorticoids, somatostatin, dopamine, prolactin, estrogen and growth hormones (Figure 1).
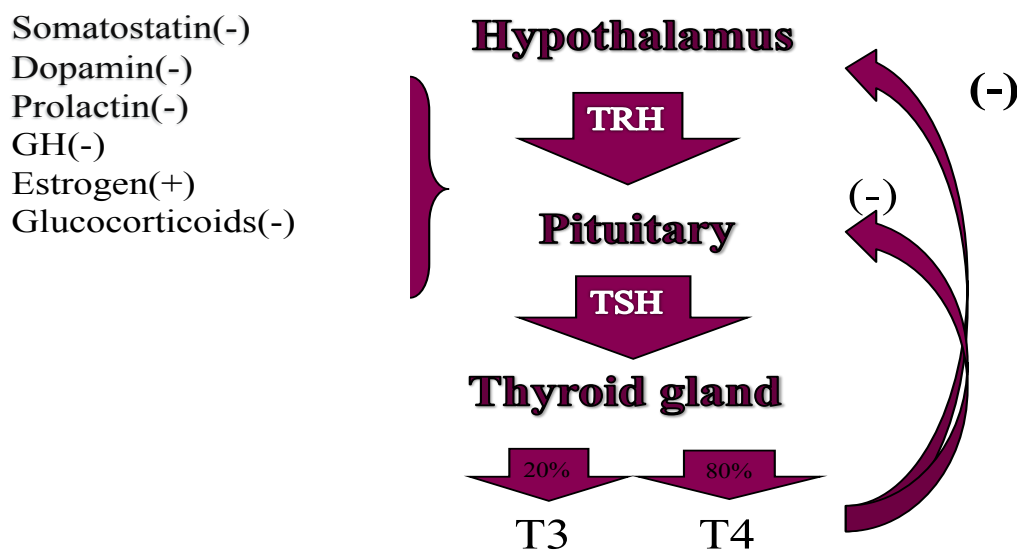
Somatostatin(-)
Dopamin(-)
Prolactin(-)
GH(-)
Estrogen(+)
Glucocorticoids(-)

Hypothalamus

TRH

(-)

Pituitary

(-)

TSH

Thyroid gland

20%   80%

T3   T4

Figure 1. The hypothalamic-pituitary-thyroid axis.

2.1.3 Thyroid stimulating hormone, TSH and TSH receptor

TSH is a heterodimeric glycoprotein hormone that shares the α-subunit with other glycoprotein hormones, such as human chorionic gonadotrophin (hCG), follicle stimulating hormone (FSH) and luteinizing hormone (LH) but it has an unique β-subunit. TSH exerts its effect by binding to the TSH-receptor (TSH R), which is located in the cell membrane of thyroid follicular cells. TSH R is a member of the G-protein associated receptor family, similar to the hCG and LH receptors [2, 3]. TSH R expression has been shown in thyroidal tissue and also in extra-thyroidal tissues such as adipose tissue, testes, ovaries and endometrium [4, 5].

2.1.4 Thyroid hormone secretion

Follicular epithelial cells located in the thyroid gland produce thyroid hormones, mainly T4. They are hydrophobic hormones that are to more than 99 % bound to proteins, mainly to thyroxine binding globulin (TBG). The free fractions of thyroid hormones (fT4, fT3), which mediate thyroid hormone action in target cells, are estimated to 0.02 % of total T4 and 0.30 % of total T3.

The local enzymatic conversion of thyroid hormone in target tissues is regulated by iodothyronine deiodinases [6]. The majority of T3 in the circulation is derived from conversion of T4 by type 2 iodothyronine deiodinases (DIO2) and type 1 iodothyronine deiodinases (DIO1). The inactivation of T4 and T3 to reverse T3 (rT3) is mediated by type 3 iodothyronine deiodinase (DIO3)[7].

Cellular transport of thyroid hormone requires active transport across the plasma membrane. This is mediated through different members of mono carboxylate transporter (MCT) and organic anion transporting polypeptide (OATP) depending on target cells[8] .

2.1.5 Thyroid hormone receptor

Thyroid hormones exert their biologic effect through thyroid hormone receptors (TRs), which act as transcription factors to regulate gene expression [9]. TRs bind to a short DNA sequence of target gene called thyroid response element (TRE), which leads to transcription. By contrast, TRs interaction with the response elements, in absence of T3 leads to suppression of basal transcriptional activity [10] (Figure 2).
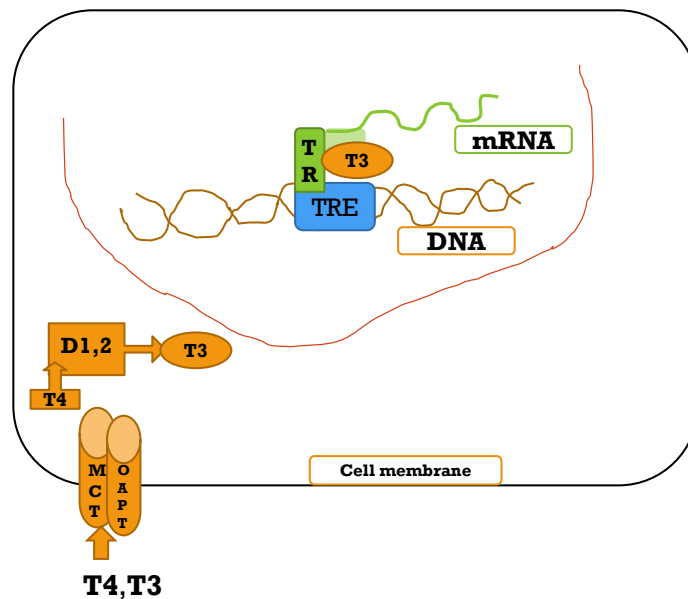
Figure 2. The action of thyroid hormone in the target cells. Thyroid hormone receptors (TRs), Thyroid response element (TRE), mono carboxylate transporter (MCT), organic anion transporting polypeptide (OATP), type 2 iodothyronine deiodinases (D2) and type 1 iodothyronine deiodinases (D1).

Thyroid receptors are encoded by two genes, TRα and TRβ, each with three isoforms; TRα1, TRα2 and TRα3 and TRβ1, TRβ2 and TRβ3 [11]. Thyroid receptors are expressed in most tissues and they have higher affinity to T3 than T4. TRα1 is predominantly expressed in brain, heart and skeletal muscles. [11]. TRβ1 is widely expressed in different organs except testes[12] . TRα1, TRα2 and TRβ1 have been shown in endometrium[4, 5].

2.2   THYROID DYSFUNCTION

Changes in serum concentration levels of TSH are the most commonly used indicator of thyroid dysfunction such as autoimmune thyroid dysfunction, hypothyroidism, subclinical hypothyroidism and hyperthyroidism.

2.2.1   Hypothyroidism

Hypothyroidism is defined as low levels of thyroid hormone combined with elevated levels of TSH. Hypothyroidism can be due to low secretion of hormone from the thyroid gland, primary hypothyroidism, or due to low levels of TSH, that is central hypothyroidism. The worldwide prevalence of hypothyroidism is between 0.6 to 12 per 1000 women and 1.3 to 4 per 1000 men [13].

Iodine deficiency is the most common cause of hypothyroidism worldwide [14, 15]. In iodine sufficient countries like Sweden, the most common thyroid disorder is chronic autoimmune thyroiditis, usually known as Hashimoto's thyroiditis. The diagnosis of Hashimoto's thyroiditis is confirmed by the presence of anti-thyroid peroxidase antibodies (TPO-Ab)[16]. Hypothyroidism can also be caused by earlier treatment of Graves´ disease such as anti-thyroid drugs, thyroidectomy or radioiodine treatment.

Symptoms of hypothyroidism are nonspecific and vary due to the severity of the disorder. Dry brittle hair and nails are common in these patients who may also have symptoms of chilliness, fatigue, weight gain and slowing of higher mental function. Treatment of hypothyroidism is thyroid hormone (L-T4) substitution.

2.2.2 Subclinical hypothyroidism

Subclinical hypothyroidism (SCH), defined as elevated serum levels of TSH combined with normal thyroid hormone levels [17]. Studies performed in the United States have shown a prevalence of 3 to 15 % of SCH. Women with SCH may have vague or nonspecific symptoms or have symptoms similar to those with hypothyroidism. Women with TPO-Ab and elevated TSH levels are at higher risk of progressing from SCH to hypothyroidism [18, 19] Women with TPO-Ab are at higher risk to development postpartum thyroiditis [20, 21].

2.2.3 Hyperthyroidism (thyrotoxicosis)

Hyperthyroidism is defined as elevated thyroid hormone levels combined with almost undetectable levels of TSH. It affects approximately 2.0 % of women and 0.2 % of men worldwide. The most common type of hyperthyroidism is Graves´ disease. This condition is due to stimulation of thyroid gland by TRAb on the thyroid follicular cells [18].

Common symptoms of hyperthyroidism are weight loss, palpitations, tremulousness, heat intolerance, and anxiety. Physical findings such as tachycardia, thyroid enlargement and tremor are also seen. Treatment options are: anti-thyroid drugs, surgery and radioiodine treatment.

2.3 THYROID DYSFUNCTION AND FEMALE REPRODUCTION

2.3.1 Hypothyroidism

Women with hypothyroidism have low levels of sex hormone binding globulin (SHBG) and low levels of estrogen and testosterone [22]. Menstrual disturbances such as oligomenorrhea, amenorrhea and menorrhagia are common in hypothyroid women. These

disturbances can partly be due to TRH-induced hyperprolactinemia and thus altered pulsatile GnRH secretion and partly due to defect hemostasis with low levels of coagulation factors. [23-25].

2.3.2 Hyperthyroidism (thyrotoxicosis)

Thyrotoxicosis may lead to different symptoms ranging from normal menstrual cycles to menstrual irregularities such as menorrhagia, oligomenorrhea, amenorrhea, anovulation and reduced fertility [26, 27] . Women with Graves´ Disease have 2 to 3 times higher serum levels of estrogen and LH during all phases of the menstrual cycle, probably due to high levels of SHBG [27] . The production of testosterone and androstenedione is also increased in these women [28].

2.4 THYROID AND PREGNANCY

2.4.1 The change in thyroid hormone production during pregnancy

In early pregnancy an estrogen derived increase in TBG (2.5-fold higher) occurs which requires an increase in thyroid hormone production and a higher daily intake of iodine (250 µg) [29-32] [33]. While the free fraction of thyroid hormone is slightly increased during the first trimester of pregnancy, the total serum levels of thyroid hormones are 1.5-fold higher in pregnant women than in non-pregnant women. The TSH levels have a transient fall during the first trimester of pregnancy due the thyrotrophic action of hCG [34], the lowest levels are seen around 10-12 weeks of gestation. In iodine sufficient areas the TSH levels will remain stable and similar to pre-gestational levels after the first trimester until the end of pregnancy.

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2.4.2 Thyroid hormones and fetus

Despite incorporation of iodine late in the first trimester of pregnancy, the fetus does not start to secrete its own thyroid hormones until 18th to 20th weeks of pregnancy. Thus, the fetus is totally dependent on the trans-placental passage of maternal thyroid hormone during the early stages of pregnancy [35, 36]. During pregnancy, the fetus can also be affected by maternal thyroid receptor antibodies and anti-thyroid drugs due to trans-placental passage (Figure 3).
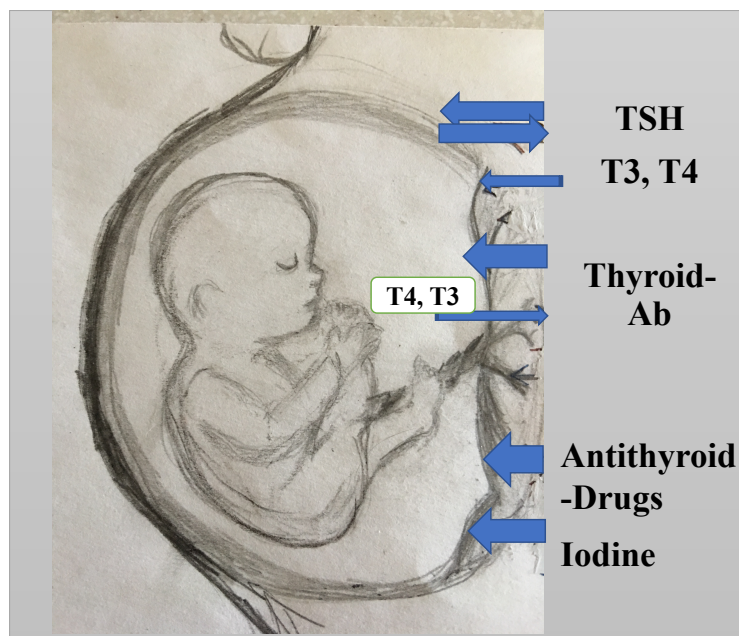

Figure 3: The trans-placenta passage of thyroid hormones and thyroid hormone related factors.

The fetus normal growth and neurologic development is dependent on optimal levels of thyroid hormones. Thyroid hormones influence neurodevelopmental events, such as neurogenesis, myelination, dendrite proliferation and synaptogenesis [37, 38].

2.5 THYROID DYSFUNCTION DURING PREGNANCY

2.5.1 Hypothyroidism/ subclinical hypothyroidism or isolated hypothyroxinemia

Untreated hypothyroidism has been associated with an increased risk of obstetric and fetal complications while this it is not the case in isolated hypothyroxinemia or SCH [39, 40] [41]. Hypothyroidism and SCH have been associated with adverse fetal and obstetric outcome such as miscarriages, preterm labor, before 32 weeks of gestation, postpartum hemorrhage, respiratory fetal distress, intrauterine growth retardation (IUGR) and neurological disorders [39, 40, 42-46]. However, there is less evidence that untreated SCH during pregnancy is associated with neurological disorders in the fetus [47].

2.5.2 Hyperthyroidism

Untreated hyperthyroidism is associated with adverse outcomes of pregnancy such as miscarriage, preeclampsia, and preterm delivery. There is a significantly higher risk of fetal complications including IUGR and congenital heart failure even in euthyroid status in the mother, due to the presence of TRAbs. Both thyroid antibodies and anti-thyroid drugs have the ability to pass through placenta [48]. Pregnancy with positive TRAbs, require careful monitoring of thyroid status and TRAbs and controls of the fetus (ultrasonography) during pregnancy. The change of the immune system during pregnancy leads to remission in Graves´ disease like many other autoimmune diseases with a risk for a postpartum relapsing [49].

2.5.3   Gestational thyrotoxicosis

The production of fT4 increases during pregnancy due to secretion of hCG, with a peak during the 10th to 12th weeks of gestation. hCG is a glycoprotein, which shares the alpha unite with TSH and can act as a TSH agonist. This leads to suppression of TSH and transient hyperthyroxinemia in the first trimester of normal pregnancy as well as multiple pregnancy and may cause hyperemesis gravidarum [50-52].

2.5.4   Postpartum thyroid dysfunction

Postpartum thyroiditis is a destructive autoimmune disease with a prevalence of 5 to 9 % and usually occurs within the first year after delivery. Women with diabetes mellitus type 1 have a threefold higher risk of developing postpartum thyroiditis. Women with positive TPO antibodies during early pregnancy have 50 % risk of developing postpartum thyroiditis and an increased risk of developing a permanent hypothyroidism [17, 53, 54]

3. ENDOMETRIUM AND FALLOPIAN TUBE

3.1 ENDOMETRIUM

The uterine wall consists of three layers; a thin lining, endometrium, a muscular layer myometrium and serosa. The endometrium, which is the innermost portion of uterus, can be divided into two layers, the upper functional layer where the implantation of blastocyst takes place and the basal layer. From the basal layer, the monthly regeneration of endometrium takes place. Histologically, the endometrium contains a layer of columnar epithelium on the surface and in the glands and a layer of stromal connective tissue. The stromal connective tissue contains fibroblasts, endothelial cells and leukocytes.

3.1.1 Endometrial differentiation during menstrual cycle

During the menstrual cycle the monthly preparation of the endometrium for implantation occurs. Differentiation of the endometrium during the menstrual cycle is a reflection of the hormones produced by the ovary [55]. The main hormones from the ovary are estrogens (E) and progesterones (P). A feedback system, consisting of hormones secreted from the hypothalamus, pituitary and ovaries regulates the menstrual cycle [56]. The menstrual cycle can be divided into three phases due to endometrial changes: one degenerative phase called menstrual phase, one proliferative phase which corresponds to the ovarian follicular phase, and one secretory phase, which corresponds to the ovarian luteal phase.

The first days of the menstrual cycle is initiated by the presence of a bleeding, due to degeneration and expulsion of the endometrium. The follicular phase is characterized by growing follicles and secretion of estrogen. During this phase, selection of a dominant follicle occurs [57, 58]. The increasing estrogen levels during the follicular phase stimulate proliferation of the endometrium, which becomes thicker and with increasing number of glands.

After the LH surge in the middle of the cycle, the dominant follicle ruptures and the oocyte is released [59]. The residual follicle rearranges to form a corpus luteum which secrets both

estrogens and progesterones [56]. During the luteal phase, the endometrium is prepared for implantation of a blastocyst. In absence of pregnancy, degeneration of the endometrium starts. After two weeks the corpus luteum forms corpus albicans. The secretion of estrogens and progesterones has dropped to low levels, which leads to degeneration of the endometrium.
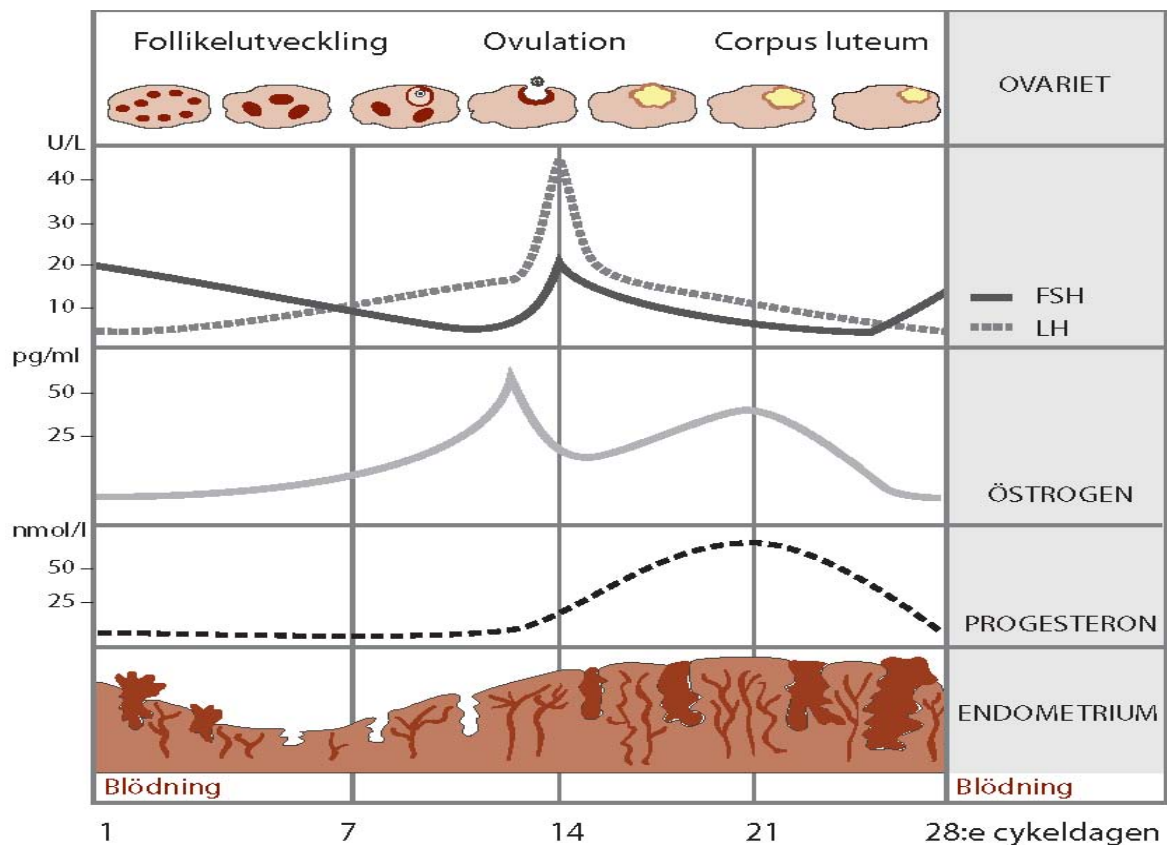
Figure 6: The menstruation cycle and its related hormones: Gonadotropic hormones; Follicle stimulating hormone (FSH), Luteinizing hormone (LH). Gonadal hormones estrogen and progesterone.

3.2   FALLOPIAN TUBE

The Fallopian tubes, are divided into four parts from the uterine horns to the ovaries; the interstitial part which transverses the uterine musculature, the isthmus, the ampulla which represents the major lateral part of tubes, where the fertilization of ovum takes place and the infundibulum with its fimbriae. The Fallopian tube wall consists of four distinct layers;

serosa, subserosa, lamina propria and mucosa. The mucosa is a single layer of cells with cilia throughout the tubes.

4. IMPLANTATION

Implantation requires fertilization of a haploid oocyte by a haploid sperm in the Fallopian tube, the development of the early embryo to blastocyst during its transport in the Fallopian tube and finally implantation of the embryo into the endometrium [60, 61]. It also includes hatching of the blastocyst at the end of the first week of embryonic development, to the formation of primitive placenta at the mid second week of embryonic development.

5. INFERTILITY

Infertility has been defined as the inability to conceive after one year or more of regular intercourses without contraception. The monthly fecundity rate in fertile couples has been estimated to 10 to 15 % [62]. The American Society for Reproductive Medicine (ASRM) and the World Health organization (WHO) considers infertility as a reproductive disease and has recommended investigation and treatment after one year for women below the age of 35 and six months for women over the age of 35. The etiology of infertility can be related to male or female factors or combinations of both. In more than 10 % of cases the etiology of infertility is unexplained [63, 64]. The female causes of infertility include age dependent factors, luteal phase defects, genetic abnormalities, anatomical abnormalities, post inflammatory factors, peritoneal factors, endometrioses and polycystic ovary syndrome.

Assisted reproductive technology (ART) involves methods used for treatment of couples with infertility such as, clomiphene citrate and intrauterine insemination, gonadotropin treatment and intrauterine insemination, in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Despite optimization of ART, all attempts to treat are not successful. A better understanding of the mechanisms involved in implantation will be beneficial for infertility treatment including increased chance of spontaneous pregnancy.

6. AIM OF THE STUDIES

6.1   GENERAL AIM

The overall aim of the studies was to explore the influence of thyroid hormone system on female reproduction.

6.2   SPECIFIC AIMS

-To detect incidence of subclinical hypothyroidism and hypothyroidism in a representative group of Swedish women in early pregnancy.

- To compare serum and endometrial levels of thyroid related factors in fertile women and women with unexplained infertility.

- To explore the presence and distribution of proteins related to thyroid, in endometrium and Fallopian tubes

- To study the influence of T4 on embryo development.

7. MATERIALS AND METHODS

7.1 ETHICAL APPROVALS

Study I was approved by the Regional Ethical Review Board in Stockholm (2009/2:6). Study II was approved by the Regional Ethical Board in Stockholm and the Regional Ethical Board in Uppsala (2008/046/1 2012/339). The women who donated Fallopian tubes and endometrium gave their informed consent before participation. The human embryos were donated for research after 5 years limit storage has passed. Both partners of the couples involved signed an informed consent form after receiving oral and written information.

7.2 MATERIAL

7.2.1 Serum samples

Serum levels of TSH in 1298 women up to 12 weeks and 6 days of pregnancy were analyzed between 2009 and 2011. The TSH levels in a high-risk group were compared to a low risk and a general screenings group (Table 1). The criteria for high-risk group wer based on recommendation for screening, in Stockholm, at the time of study. Women with diabetes sever cardiovascular diseases and systemic lupus, were not included in this study, since they are usually attend to specialist care unites.

Furthermore *serum levels of* fT4, TSH and TPO-Ab in women with proven fertility were compared to women with unexplained infertility.

7.2.2 Endometrial and Fallopian tube biopsies

Endometrial biopsies from women with unexplained infertility was compared to endometrial biopsies from healthy fertile controls for detection of TRα1, TRβ1, TSH R, DIO2 and MCT8 proteins. These samples were obtained during receptive phase (Table 1).

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Fallopian tube samples were used for analyses of TRα1, TRβ1, TSH R and DIO2. These samples were collected from healthy volunteers with proven fertility attending the hospital for laparoscopic tubal sterilization or hysterectomy due to myoma. Six samples were taken during the follicular phase, cycle day (cd) 1-13, and seven samples were during the luteal phase, cd 14-28 (Table 1).

Endometrial biopsies were used for detection of MCT8 protein in fertile women (n = 28) with an average age 35.9 years and the mean BMI of 24.9. These samples were taken during follicular phase on cycle days 1 to 13 (n = 9), early luteal phase on cycle days 14 to 18 (n = 6), mid luteal phase on cycle days 19 to 22 (n = 6) and late luteal phase on cycle day 23 to 28 (n = 7).

Table 1. Characteristics of study participants included in study. Data is presented as median and median - range. Statistics according to Chi Square, Mann-Whitney rank sum test and Kruskal Wallis test. Statistically significance value was set at $p \leq 0.05$.

TSH samples	Low risk (n=511)	High risk (n=88)	General (n=699)	P-Value
Age	33 (17-49)	35 (22-44)	30 (16-50)	0.001
BMI	23 (17-46)	23 (15-34)	-	0.718
Serum samples	**Fertile (n=67)**	**Infertile (n=147)**		
Age	36 (24 - 44)	34 (22 – 40)		0.899
BMI	23.3 (18.4 – 32.8)	22.8 (17.1 – 37.4)		0.152
Endometrial samples	**Fertile (n = 29)**	**Infertile (n = 19)**		
Age	36 (25 – 41)	35 (31 – 39)		0.208
BMI	24.5 (22.5 – 27.4)	22.3 (18.4 - 29.4)		0.475
Tubal samples	**Follicular phase (n=6)**	**Luteal phase (n=7)**		
Age	40 (37 – 44)	41 (39 – 46)		0,295
BMI	28.5 (23.1 – 36.8)	28.2 (21.4 – 35.5)		0.413

7.2.3 Human embryos

Human embryos were used to explore addition of T4 (n = 38) compared to control embryos (n = 36) on early blastocyst development. The blastocysts were scored according to Gardner and Schoolcraft [65]. Each embryo is assigned a quality score based on blastocyst development, quality of inner cell mass and quality of trophectoderm.

7.3 METHODS

7.3.1 ELISA

Serum samples from pregnant women were collected at the first visit at maternal care units and TSH in serum was analyzed according to clinical routine at Karolinska University Hospital in either Solna or Huddinge.

All blood samples in fertile and infertile women were collected at the clinic or at the research laboratory and were stored at -20°C. All samples were analyzed at the same times by use of ELISA according to instruction from manufacturer. Enzyme linked immunosorbent assay (ELISA) were used for analyzing of fT4, TSH and TPO-Ab.

7.3.2 Embryo Culture

Frozen-thawed embryos of high quality embryos were used. The embryos were randomly allocated to be cultured in standard media with T4 added to a final concentration of 20 pmole/L (n=38) or in standard culture media (n= 36) until day 6 and thereafter blastocyst formation was determined according to Gardener.

7.3.3   Endometrial and Fallopian tube samples

The endometrial biopsies were obtained using pipelle aspiration while Fallopian tube samples were obtained during surgery. For determination of the day of cycle, either the LH surge or cycle day was used. The tissue samples were fixed in 4 % formaldehyde and then stored in 70 % ethanol until embedding.

7.3.3.1   Immunohistochemistry

Immunohistochemistry (IHC) was used for detection and location of thyroid related proteins in tissue from endometrium and Fallopian tube. Immunohistochemistry is a method used primarily to detect the distribution of proteins in a tissue. In brief, a specific primary antibody interacts with an antigen in the tissue (Table 3). For detection a biotinylated secondary antibody conjugated and thereafter visualized by enzymatic activation of a chromogenic substrate was used.

Table 3. Antibodies used in immunohistochemistry.

Antibody	Receptor	Concentration
Polyclonal Rabbit IgG	TRα1	5 µg/ml
Polyclonal mouse IgG	TRα2	5 µg/ml
Polyclonal Rabbit IgG	TRβ1	3 µg/ml
Monoclonal mouse IgG	TSH R	2.5 µg/ml
Polyclonal Rabbit IgG	DIO2	50 µg/ml
Polyclonal Rabbit IgG	MCT8	50 µg/ml

For evaluation of immunohistochemistry, two observers evaluated the staining intensity of each sample. Staining intensity and the number of stained cells were graded according to the following: 0 = no staining, + = faint staining, ++ = moderate staining, and +++ = strong staining.

7.3.4 Statistical Analysis

For descriptive statistics, independent t-test or Mann-Whitney Rank Sum Test were used. For comparisons between three or more groups, chi-square test was used in the first study and Kruskal-Wallis test followed by Dunn's test was used in the second study. For the number of median and good quality embryos, Fischer's exact test was used. All statistical differences with p values ≤ 0.05 were considered statistically significant.

8. RESULTS

The incidence of subclinical hypothyroidism and hypothyroidism was almost the same, (almost 10), in all three groups regardless of TSH levels ≥ 2.0 mU/L or ≥ 2.5 mU/L in early pregnancy (Figure 5).
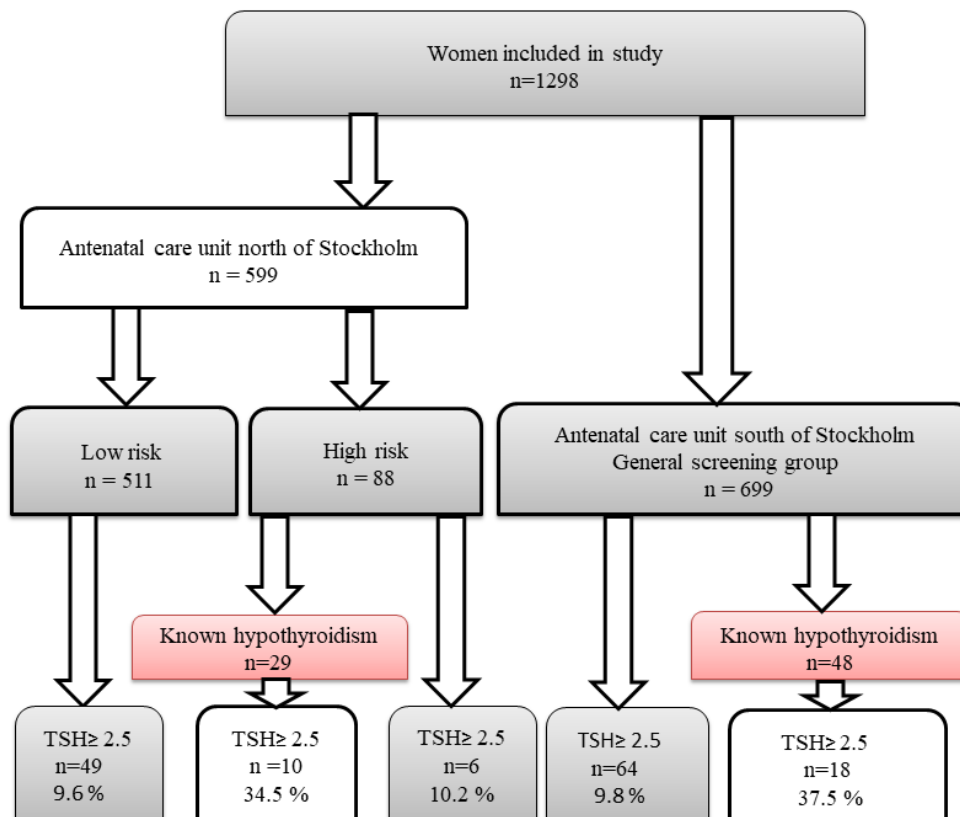

Figure 5. Flow diagram of study population of early pregnant women and the incidences of subclinical hypothyroidism and hypothyroidism

Hypothyroid women on LT4 treatment prior to pregnancy (n= 77) had in 50.6 % of cases a TSH level ≥ 2.0 mU/L and in 36.4 % of cases a TSH levels ≥ 2.5 mU/L.

During this study all women with subclinical hypothyroidism were treated with LT4 and monitored with control of fT4, TSH during pregnancy with a goal of treatment at TSH level < 2.0 mU/L.

8.1.1 Serum level of TSH, free T4 and TPO-Ab in fertile and infertile women

The women with unexplained infertility had significantly higher serum level of fT4. There were no significant differences in the serum levels of TSH and TPO-Ab between the groups (Figure 6).
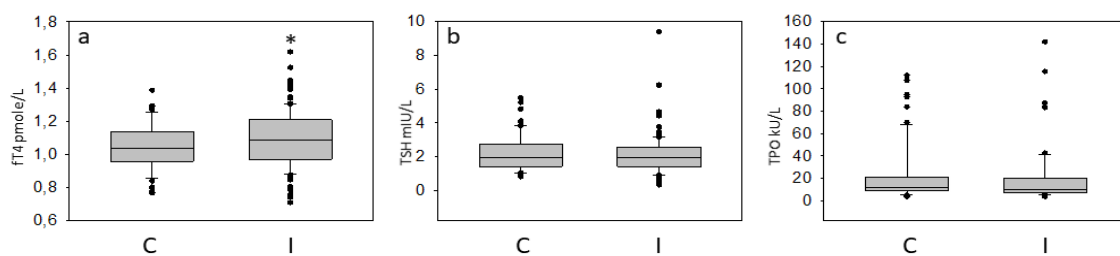

Figure 6. Serum levels of fT4, TSH and TPO-Ab in study population: Serum levels of (a) fT4 and (b) TSH and (c) TPO-Ab in fertile control women (C) and in women with unexplained infertility (I). Statistics was performed according to Mann- Whitney Rank Sum Test, p < 0.05 was considered statistical difference (*).

8.1.2 Thyroid hormone influence on embryo development

The number of good quality embryos, according to Gardner and Schoolcraft's classification, were significantly higher (p = 0.031) in the T4 treated group, 25 (65 %), compare to embryo in control group 18 (50 %). (Figure 7)
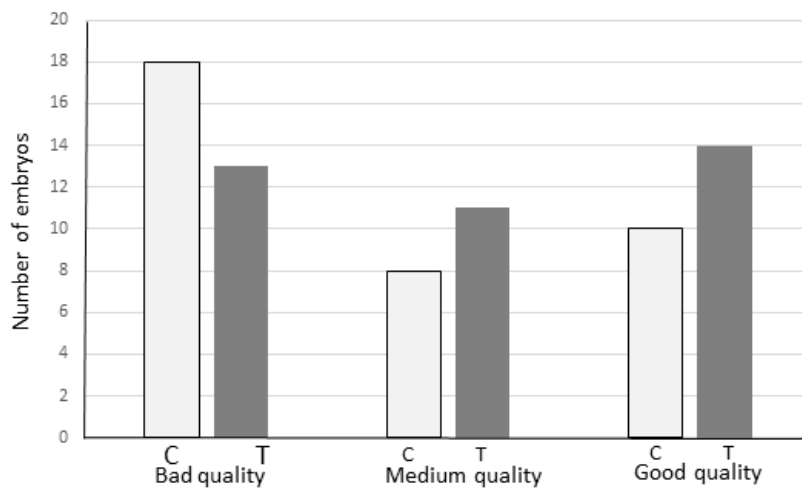
Figure 7. Number of embryos that reached a good quality blastocysts in (C) Control group and in (T) Treated with thyroid hormone Statistics according to Fischer's exact test calculated on the percentage for the number of median and good quality embryos, $p < 0.05$ was considered statistical difference.

8.1.3 The protein staining of

8.1.4 R, TRα1, TRβ1, DIO2and MCT8 in the endometrium in infertile and fertile controls.

The highest protein staining of TSH R, TRα1 and TRβ1 was identified in the luminal epithelium. There was a significant lower protein staining of TRα1 and MCT8 in glandular epithelium in endometrium from unexplained infertile women compared to endometrium from fertile controls. (Figure 8)
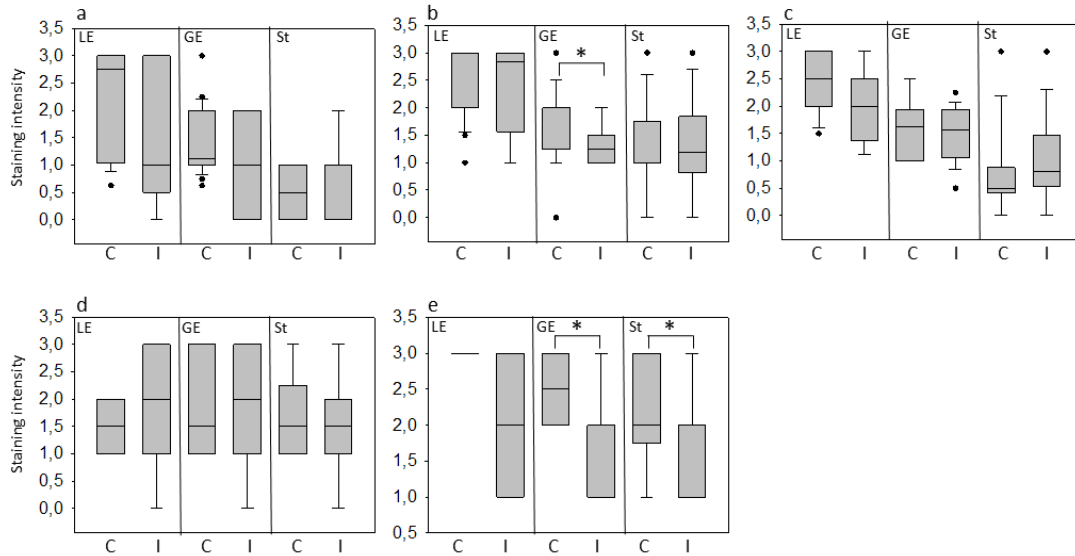
Figure 8. Endometrial protein staining of TSH R (a), TRα1 (b), TRβ1 (c), DIO2 (d) and MCT8 (e) in endometrium from women with unexplained infertility compare to controls. The staining intensity was generally higher in the luminal epithelium (LE) followed by the glandular epithelium (GE) and the least intense staining was seen in the stroma (St). Statistical differences were calculated according to Kruskal-Wallis U-test, p < 0.05 was considered statistical difference (*).

8.1.5 The protein staining of MCT8 in endometrium during the menstruation cycle in fertile women

The protein staining of MCT8 showed to be significantly higher in mid luteal phase in glandular epithelium in fertile women (Figure 9).
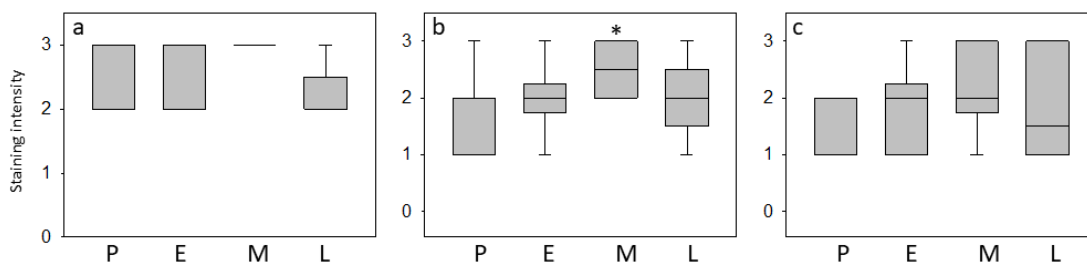

Figure 9. The protein staining of MCT8 in the endometrium during the menstrual cycle. The luminal epithelium (a), glandular epithelium (b) and stroma (c). The most intense staining intensity was seen in the mid luteal phase (M) compared to the follicular phase (P), early luteal phase, (E) and late luteal phase (L). Statistical differences were calculated according to Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's test. p < 0.05 (*) was considered statistical difference.

8.1.6 The protein staining of TSH R, TRα1, TRβ1 and DIO2 in the Fallopian tube

Furthermore we could identify staining of proteins related to thyroid in Fallopian tube. The TSH R was identified in the cytoplasm of epithelium and muscle cell in isthmus and fimbria and in all three-cell types in the ampulla part of Fallopian tubes. The TSH R, TRα1, TRβ1 and DIO2 proteins were identified in the cytoplasm of epithelium, as well as in the stromal and vessels in all three part of the Fallopian tube. The highest staining of these proteins was identified in the epithelium, p <0.001(Figure 10).
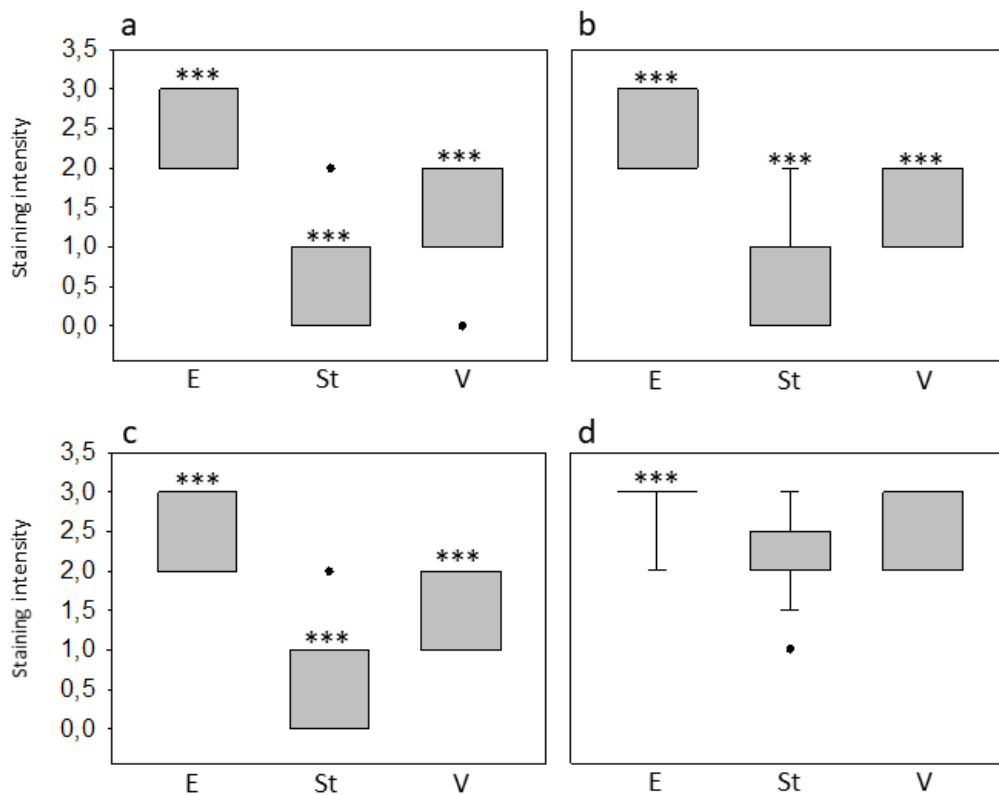

Figure 10. The immune staining intensity of TSH R (a), TRα1(b), TRβ1 (c) and DIO2 (d) in the three compartments of Fallopian tube from fertile women. The staining intensity of TSH R, TRα1, TRβ1 and DIo2 was significantly different in all three compartments of fallopian tubes; Epithelial cells (E), Vessels (V), Stroma (St). Statistics according to Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's test, p < 0.001 (***) was considered statistical difference.

9. DISCUSSION

It is well known that thyroid dysfunction have negative influence on female fertility and there is growing evidence of a correlation between untreated thyroid dysfunction during pregnancy and adverse pregnancy outcome for the pregnant woman and the growing fetus [40, 44, 46, 53, 66]. Even the association between SCH with infertility and adverse pregnancy outcomes is discussed, it's clear that untreated hypothyroidism and hyperthyroidism are associated with pregnancy complication. This highlights the importance of proper screening model for detection of thyroid dysfunction in early pregnancy.

Our study showed almost the same incidence of SCH and hypothyroidism (9.6 %-10.2 %) in all pregnant women regardless of risk of thyroid disease. This confirms the results of a previous study conducted in Uppsala community in Sweden [67], which showed a similar incidence of elevated trimester specific TSH in a target screening group (12.1 %), and in an unselected screening group (12.6 %). Furthermore, a Cochrane review of different screening methods of TSH during early pregnancy showed that the number of women diagnosed and subsequently treated for thyroid dysfunction will increase by universal screening [68].

This study highlights the importance of analyzing TSH as a measure of thyroid function in women with hypothyroidism on LT4 treatment prior and early in pregnancy. In our study, in 50 % of cases the women on LT4 treatment had suboptimal LT4 treatment in early pregnancy. This is in agreement with studies by Hallengren et al. in Malmö, Sweden and Vadiveloo et al. in Tayside, Scotland, who reported that 30 % of women on LT4 treatment had suboptimal treatment [69, 70]. The reason why this is important is that the maternal production of thyroxin increases by 30 %–50 % in early pregnancy. Therefore, women with known hypothyroidism usually need increased dose of LT4 as soon as pregnancy is confirmed. The fetal secretion of thyroid hormone does not start until 18th to 20th weeks of pregnancy. Thus, it is of importance to achieve adequate thyroid hormone levels in these women during early pregnancy [71].

There is an ongoing debate about the upper limit of the normal TSH and the benefits of thyroxine therapy, in case of SCH, during early pregnancy. The cut-off TSH level (TSH 2.0 mU/L) was based on clinical guide lines for treatment of LT4 during pregnancy at the time of study in Stockholm. In 2017, the previous recommendation for upper limit of serum TSH

level of first trimester was specified by the American Thyroid Association from TSH 2.5 mU/L to 3.5 mlU/L [72]. The Swedish guidelines for treatment of SCH during pregnancy now include an upper limit of TSH at 3.5 mU/L during the first trimester and general screening during early pregnancy has been recommended (http://online.liebertpub.com/doi/ pdfplus/10.1089/thy.2016.0457). However, general screening for thyroid dysfunction has not yet been recommended by the American Thyroid Association.

In the present study, the women with unexplained infertility showed a slightly higher level of fT4, reduced protein staining of TRα1 and MCT8. The lower levels of TRα1 in the endometrium of infertile women, might explain the required higher levels of fT4. Thyroid hormone supplementation in rat has induced higher expression of TRα and TRβ in the uterus [73].

In addition, lower levels of MCT8 were found in both endometrial glandular epithelial cells and stromal cells from infertile women compared to fertile control women. The action of thyroid hormone at the target cell level is dependent on the ability of T4 and T3 transport into the cells, local enzymatic activation of fT4 and co-regulators. The reduced levels of TRα1 on the endometrial cell in women with infertility might be the results of low levels of thyroid hormone in these cells, due to reduced levels of MCT8.

Furthermore, the present study showed higher levels of MCT8 protein during the mid-luteal phase, which indicates the ability to transport TH into the epithelial cells might be important for normal receptivity of the endometrium.

The presence of TH in culture media had a positive impact on embryo development in vitro. Furthermore, the presences of different proteins belong to direct action of thyroid system on Fallopian tubes, implicating a physiological role in regulating early embryo development.

In the present study it was demonstrated that by adding T4 to culture media, embryo development was enhanced. Previous studies in bovine and mouse have shown the same improvement of thyroid treatment for early embryo development. Furthermore, in a systematic review of LT4 treatment in women undergoing IVF/ICSI was shown an improvement of take-home baby rate in this group. Therefore, LT4 supplementation for women with SCH and/or thyroid autoimmunity who are undergoing fertility treatment is recommended.

One limitation of this study was that TSH and fT4 levels in serum were not obtained during sampling of endometrium.

In conclusion, adequate diagnosis and treatment of thyroid disease in women that are either pregnant or trying to become pregnant is of importance for successful pregnancy outcome.

10. CONCLUSIONS

• High-risk screening is not optimal to determine which women are at risk of thyroid disease during early pregnancy.

• Early adjustments of thyroid hormone treatment is important in case of known hypothyroidism in women during early pregnancy.

• Thyroid systems in women with infertility (fT4 level, TRα1and MCT8) seem to be imbalanced.

• The presence of TRα1, TRβ1 and TSH R, and the thyroid hormone related proteins, MCT8 and D2 in the Fallopian tubes in combination with improvement of embryo development by T4 treatment show that thyroid hormones are important for early embryo development.

• Supplementation of thyroid hormone in preimplantation embryo culture seem to improve embryo development and might thereby increase success rate after fertility treatment.

11.   ACKNOWLEDGEMENTS

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