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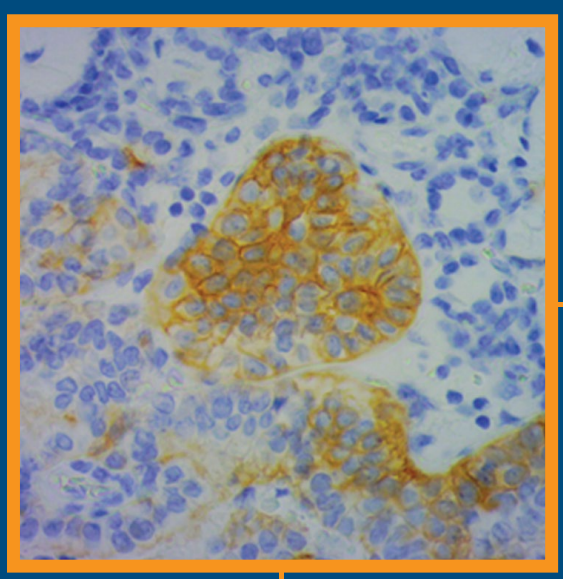

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Illustration on the cover: *CK19 immunoreactivity of papillary thyroid cancer in struma ovarii* (see: Ewelina Szczepanek-Parulska et al., pp. 35–41)



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REVIEW

VIA MEDICA

# *In vitro* generation of pancreatic $\beta$-cells for diabetes treatment. I. $\beta$-like cells derived from human pluripotent stem cells

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**Abstract**
Diabetes mellitus is a chronic disease that affects hundreds of millions of people worldwide. Type 1 diabetes (T1D) is characterized by the lack of pancreatic $\beta$-cells that had been destroyed as a result of an autoimmune response. Therefore, in patients with T1D, the replacement therapy with functional $\beta$-cells derived from extrinsic sources could be a preferable option as compared to insulin treatment. Unfortunately, successful transplantation of whole pancreata or pancreatic islets into patients with diabetes is available only to a fraction of them due to the scarcity of donors. The rapid development of cell reprogramming methods made it possible to generate large numbers of human $\beta$-like cells derived from human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs). This review describes the basis of *in vitro* differentiaton protocols of $\beta$-like cells that mimic changes of the main signaling pathways during the key stages of human and murine pancreas development, which are described first. During the last 15 years it was found that there are no important differences between hESCs and hiPSCs in their differentiation capacities into $\beta$-like cells and the expression profiles of the key transcription factors. The *in vitro* produced $\beta$-like cells are immature as demonstrated by functional tests in rodents and single-cell transcriptomic and proteomic analyses. After the transplantation of the $\beta$ cell progenitors into immunocompromised diabetic mice, a few weeks have to pass before the increased insulin levels in response to glucose load appear. There is a continuous progress in the development of open-type encapsulation devices which allow the vascularization of the transplanted cells and protect them against host's immune cells. The results of the first clinical trial of human partially differentiated endocrine progenitors of $\beta$ cells transplanted into patients with T1D will be published in the year 2019. It is hoped that further improvements in the techniques of large-scale generation of the $\beta$-like cells derived from human pluripotent stem cells will bring us closer to their clinical application as a form of cause-directed therapy for people with diabetes. *(Folia Histochemica et Cytobiologica 2019, Vol. 57, No. 1, 1–14)*

**Key words:** diabetes; human pancreas development; hESC; hiPSC; *in vitro* differentiation; transcription factors; $\beta$-like cells; $\beta$ cell replacement

## Abbreviations*:

Arx — aristaless-related homeobox; bFGF — basic fibroblast growth factor; bHLH — basic helix-loop-helix family; BMP — bone morphogenetic protein; Cdx2 — caudal-type homeobox transcription factor 2; c-Myc — v-Myc avian myelocytomatosis viral oncogene homolog; CPA — carboxypeptidase A; CXCR4 — chemokine (C-X-C motif) receptor 4; DE — definitive endoderm; DLL1 — delta-like canonical Notch ligand 1; Dpc — day post conception; EP — endocrine progenitor; EGF — epidermal growth factor; ESC — embryonic stem cell; Ex4 — exendin-4; FACS — fluorescence-activated cell sorting; FGF — fibroblast growth factor; FoxA1, -2 — forkhead box A1, -2; Gata4 — GATA-binding protein 4; Gata6 — GATA-binding protein 6; Glis — Gli similar family of transcription factors; GSIS — glucose-stimulated insulin secretion; GSK3$\beta$ — glucagon synthase kinase 3$\beta$; hESC — human embryonic stem cell; Hnf4 — hepatocyte nuclear factor 4; Hnf6 — hepatocyte

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nuclear factor 6, synonym — Onecut1; hPSC — human pluripotent stem cell; hiPSC — human induced pluripotent stem cell; IDE1/2 — inducers of definite endoderm 1/2; Idx1 — islet/duodenum homeobox 1, synonym of Pdx1; IGF-1 — insulin-like growth factor 1; Ins — insulin; Ipf1, insulin promoter factor 1, synonym of Pdx1; iPSC — induced pluripotent stem cell; Irx1 — iroquois homeobox protein 1; Isl1 — LIM homeobox 1; KGF — keratinocyte growth factor; Klf4 — Krüppel-like factor 4; Ldb1-Isl1 — LIM domain-binding protein 1–islet 1; MafA, -B — avian musculoaponeurotic fibrosarcoma oncogene homolog-A or -B; Mesi1 — Meis homeobox 1; Mist1- bHLH family member, a15; N0); Mnx1 — motor neuron and pancreas homeobox 1; MPC — multipotent progenitor cell; MPP — multipotent pancreatic progenitor; NeuroD1 — neurogenic differentiation factor 1; Neurog3 — neurogenin 3; Ngn3, acronym for Neurog3; Nkx2.2 — Nirenberg and Kim homeoboxfactor 2.2; Nkx6.1 — Nk6 homeobox protein 1; Oct4 — octamer binding transcription factor 4; Onecut — synonym of Hnf6; Pax4, -6 — paired box homeodomain transcription factor 4, -6; Pdx1 — pancreatic and duodenal homeobox 1 transcription factor; PKC — protein kinase C; PSC — pluripotent stem cell; Ptf1a — pancreas transcription factor 1 subunit alpha; RA — retinoic acid; Rbpj — Ras-associated protein Rap1; Rfx — regulatory factor X; Shh — sonic hedgehog; Sox9, -17 — sex-determining region Y box 9, -17; SUSD2 — Sushi domain containing 2; T1D — type 1 diabetes; Tcf1 — transcription factor 12; TGF-$\beta$ — transforming growth factor $\beta$; Wnt3 — wingless-type MMTV integration site family, member 3.

## Introduction

Diabetes mellitus (DM) is a chronic metabolic disease that currently affects more than four hundred millions of people worldwide [1]. DM is caused by a nearly absolute lack of insulin in type 1 diabetes (T1D) patients or an insufficient release of insulin in type 2 diabetes (T2D) related to insulin resistance of peripheral tissues, mainly, but not only, due to increased body and fat mass in obese patients. T1D is usually caused by autoimmune mechanisms that result in the reduction of $\beta$ cells' number in pancreatic islets. The pathogenesis of T2D is based on the overload of $\beta$ cells by the resistance of the peripheral tissues, such as skeletal muscles and fat cells, to insulin's action caused by increased serum levels of free fatty acids and oxidative stress in pancreatic islet cells. Although for almost one hundred years exogenous insulin has been administered to T1D patients as a substitute of the endogenous hormone, the lifelong treatment does not affect the cause of the disease and may be associated with many complications of which episodes of hypoglycemia are most important. T2D is treated by drugs that increase the secretion of insulin, its tissue sensitivity, improve glucose disposal, or delay glucose absorption in the gastrointestinal tract. However, in many T2D patients the exhaustion of $\beta$ cells eventually occurs and leads to the additional treatment with insulin. The best causative treatment for T1D would be to increase the number of functional $\beta$ cells. This has been in fact achieved in a relatively small number of patients, either through transplantation of cadaver-derived whole pancreas, pancreatic islets, or infusion of isolated $\beta$ cells into gastrointestinal venous system of recipients. These treatment options are available in many developed countries; however, to a limited extent only. The main limitation, similarly as with transplantation of other organs, is the scarcity of donor tissues, which makes the allotransplantation options practically unavailable to the majority of patients with DM. Moreover, the transplanted islets require lifelong immunosuppression which poses further health problems.

The continuous progress of molecular and cell biology techniques makes it possible to generate *in vitro* human functional $\beta$ cells and introduce them into diabetic patients to increase endogenous insulin production in response to changes in blood glucose concentrations. In this short review we will describe the methods that have been developed not only to produce *ex vivo* $\beta$-like cells but also to efficiently protect them from an immune attack after transplantation into diabetic patients (the term '$\beta$-like cell' is here defined as an insulin-positive cell that contains secretory granules; however, it does not imply presentation of the same functional characteristics as primary human $\beta$ cell). There are two main sources for the *in vitro* generation of human $\beta$-like cells: (i) human embryonic stem cells (hESCs) and human induced pluripotent stem cells, both referred further as pluripotent stem cells (hiPSCs), and (ii) differentiated cells from various organs that can be transdifferentiated into $\beta$-like cells, mainly by genetic manipulations. It seems obvious that the obtained $\beta$-like cells should express molecular markers of normal $\beta$ cells such as a set of specific transcription factors and other molecules that characterize mature $\beta$ cells as a result of terminal differentiation. Moreover, after transplantation into animals with experimentally-induced diabetes or into patients with diabetes, these *ex vivo* generated $\beta$-like cells should present features of functional maturity such as rapid alterations of intracellular $Ca^{2+}$ concentration and secretion of insulin in response to changing glucose levels.

*Since originally most of the transcription factors have been detected and described in *Drosphila* and non-human vertebrates, their abbreviations are written in small letters.

The elucidation of the molecular background of the stages of pancreas development in mouse and humans laid solid foundations for the implementation of cell and molecular biology techniques to transform hESC, hiPSC, or normal somatic cells into functional $\beta$-like cells, processes often termed collectively as 'cell reprogramming'. This paper will provide an overview of the progress in the field of the *in vitro* $\beta$ cell neogenesis from pluripotent stem cells as a potential source of insulin-secreting cells for patients with diabetes. Since the protocols for obtaining *in vitro* $\beta$-like cells from human PSCs to a great extent recapitulate the embryonic stages of pancreas development, a short review of the factors that control the development of human (and murine) pancreas, and especially $\beta$ cells, will be first provided.

## The embryonic development of the pancreas and its driving forces

The development of pancreas is, in general, very similar in many vertebrate species. Since the availability of early human embryos for studies is limited, the knowledge about the mechanisms of the embryonic development of pancreas in mouse (and other vertebrates) could be generally transposed to the early stages of pancreatic development in humans. The genetic manipulations in mice allowed for the identification of multiple transcription factors which in a complex but coordinated way control the development and growth of the pancreas. Many, although not all, of these transcriptions factors were shown to play a key role in human pancreatic development. Moreover, in transgenic mice it was possible to perform lineage tracing experiments that enabled to characterize the developmental history of the major cell types of endocrine and exocrine parts of the pancreas [2]. This knowledge has been used by many groups to mimic *in vitro* the developmental pathway of $\beta$ cells in a step-wise differentiation of hESC or hiPSCs into insulin-secreting $\beta$ cells, and also other pancreatic islets' cell types of which $\alpha$, $\delta$, $\varepsilon$, and PP cells secrete glucagon, somatostatin, ghrelin, and pancreatic polypeptide, respectively.

In mammals pancreatic development begins in two distinct areas that later fuse in one organ. In mouse during gastrulation the flat definitive endoderm folds to form primitive gut tube in which foregut, midgut, and hindgut can be recognized. The foregut is divided into the anterior part that gives rise to the thymus, thyroid, parathyroid, and lungs, and the posterior foregut. The evaginations of the posterior foregut appear at the embryonic day 9 (E9d) as a dorsal pancreatic bud (at the junction with midgut) and as the ventral bud at E9.5d [3, 4]. Due to the gut rotation at E12–13d, the ventral and dorsal buds become adjacent and fuse with each other. There are two developmental phases during pancreas development in mouse. During the primary transition (E9.5–12.5d) there is massive proliferation of pancreatic progenitor cells forming stratified epithelium in which several micro-lumen structures develop that later fuse [3, 5–7]. At this stage few endocrine cells appear that are mainly polyhormonal, expressing both glucagon and insulin, and some monohormonal cells; however, these cells probably do not commit to mature islets [3, 8]. During the secondary transition from E13.5d to E16.5d microlumens fuse to create the characteristic tubular structures lined by pancreatic epithelium in a form of plexus that branches further into a continuous epithelial network, segregated into tip and trunk domains [7, 9, 10]. At this stage of pancreatogenesis the proliferation of endocrine progenitor cells is extensive and all five types of endocrine cells are generated [3]. The pancreatic acini originate from distal tip cells whereas the trunk cells will differentiate into ductal and endocrine cells [3, 7, 11]. The extensive proliferation of pancreatic epithelium is accompanied by the differentiation of endocrine progenitors into neurogenin 3 (Ngn3)-positive cells followed by the formation of mature endocrine cells [12–14].

In comparison to the bulk of data on early embryogenesis in model non-human species such as mouse and chick, the information about the early stages of human development, and of pancreas in particular, is limited, mainly due to the scarcity of the early human embryos (< 8 weeks of gestation, G8w) available for investigations. However, similarly as in mouse, the differentiation of the human endoderm into $\beta$ cells can be classified into the following pivotal stages: (i) definitive endoderm (DE), (ii) primitive gut tube, (iii) foregut endoderm, (iv) pancreatic anlagen composed of multipotent progenitor cells (MPCs) that will differentiate into the three major pancreatic cell lineages, *i.e.* exocrine, ductal, and endocrine cells, (v) endocrine progenitors, and (vi) $\beta$ cells [15–18].

Pan and Brissova [16] and Jennings *et al.* [17] compared the stages of human pancreas development, its key features, and estimation of an equivalent timeline of the mouse pancreas development. At the gestational day 25–27 (G26–27d), *i.e.* at the Carnegie stages 9–10 (CS9–10), the transient contact of the notochord (and dorsal aortae) with the pre-pancreatic endoderm suppresses the expression of the sonic hedgehog (SHH) pathway. This induces folding of the

posterior foregut-derived definitive endoderm which results in the formation of the pancreatic dorsal bud at G26d that is followed by the formation of two ventral buds at G30d (CS12–13) [15, 17]. The left ventral bud gradually regresses and the right ventral bud migrates backwards at G35d (CS15) as a result of gut rotation and finally fuses with the dorsal bud at gestational weeks 6–7 (G6–7w, CS18–20) to form a single organ with the main pancreatic duct formed by the coalescence of the dorsal and ventral ducts [15]. In humans the dorsal pancreatic bud will form the major part of the head, the body, and the tail of the future pancreas whereas the right ventral bud will form the inferior part of the head and uncinate process [4]. Between G35d and G45d (CS15 and CS19, respectively) an extensive growth of the pancreatic anlagen leads to the formation of ductal branching and appearance of microlumens in the acinar cell compartment as well as extensive proliferation of multipotent progenitor cells. At G7w (CS19) tip-trunk compartmentalization becomes distinguishable: populations of tip-like cells will differentiate into exocrine acini whereas the trunk-like cells will develop into endocrine and ductal cells [16, 17].

These morphogenetic processes are initiated and controlled by transcription factors and signaling molecules that activate or suppress basic signaling pathways such as WNT, SHH, and Notch (discussed in 11, 15, 18, 19). At G30d (CS12) the expression of the crucial transcription factor PDX1 (pancreatic and duodenal homeobox 1), the earliest pancreas-specific transcription factor, can be detected in the endoderm differentiating into pancreatic anlagen [15], similarly as during mouse embryogenesis in the pancreatic endoderm [5, 6, 11]. Soon thereafter, the expression of other transcription factors: SRY (sex-determining region Y)-box 9 (SOX9), GATA4 (Gata-binding protein 4), and NKX6.1, necessary for human pancreatic development, takes place [15, 20].

Similarly as in mouse, the expression of the NEUROG3 transcription factor is a sign of the beginning of the endocrine commitment [21] that is further controlled by the expression of other transcription factors. At G7.5w (CS21) the first endocrine, insulin-positive cells appear, and at G9w glucagon–expressing $\alpha$ cells emerge [15, 21–23]. At G10w the delamination and endocrine cell clustering starts so that at G12-13w all types of endocrine cells are present in the developing islets [15, 16, 21, 22].

The role of numerous transcription factors that induce and control the development of the pancreas by the regulation of gene expression during the subsequent stages of pancreas development has been in depth characterized in mouse [11, 18, 19, 24]. They were found, although with few exceptions, to play a similar role during the development of human pancreas [15, 17, 25]. In mouse the differentiation of the multipotent progenitor cells into exocrine, endocrine, and ductal lineages of the adult pancreas is regulated by the coexpression of the key transcription factors such as Pdx1, Ptf1a, Foxa2, Sox9, Nkx6.1, and the presence of carboxypeptidase 1A (Cpa) [6, 11, 18, 24]. The development of the ductal/endocrine lineages from MPCs is associated with the loss of Ptf1a and maintenance of Nkx6.1 expression, whereas the development of the exocrine compartment depends on the downregulation of Nkx6.1 and continued expression of Ptf1a [26]. The differentiation of $\beta$ cells is controlled by the Pdx1 transcription factor which promotes specification and maturation of MPCs into pancreatic $\beta$ cells in concert with the other transcription factors such as neurogenin 3 (Ngn3), Nkx6.1, and MafA [6, 18]. Many of the transcription factors that control the development of the murine pancreas were identified during the stages of human pancreas differentiation [13, 15, 25] and are shown in Figure 1. The role of some transcription factors that are crucial for the pancreatogenesis will be shortly characterized.

In humans PDX1, also called insulin promoter factor 1 (Ipf1) or islet/duodenum homeobox 1 (Idx1), a marker of early MPCs, is detected at G30d (CS12) in a presumptive pancreatic endoderm even before pancreatic bud formation from definitive endoderm takes place [15]. PDX1 plays a pivotal role in the differentiation of all pancreatic cell types, as Pdx1 knockout mice exhibit pancreatic agenesis [27]. The role of PDX1 in the human pancreatic development was confirmed when a neonate presented lack of exocrine pancreas function and insulin-requiring hyperglycemia caused by a homozygous point deletion resulting in a frame shift [28]. Direct lineage tracing studies in mouse showed that Pdx1-expressing progenitors in the early mouse embryo give rise to all pancreatic cells types, *i.e.* ductal, exocrine, and endocrine cells, and that the progenitors for the mature pancreatic ducts separate from the endocrine/ /exocrine tissues before the E12.5 [2]. In the pancreatic endocrine cells Pdx1 is expressed mainly in the $\beta$ cells where it binds to and activates the insulin promoter and increases insulin gene transcription. Larsen and Grappen-Botting [11] presented a graphical overview of the interconnectivity of the pancreatic progenitor gene regulatory network at the early stages of mouse pancreatic development, with the Pdx1, Ptf1a, Foxa2, and Sox9 transcription factors as the key nodes. A recent genome-wide analysis of the *in vitro* generated

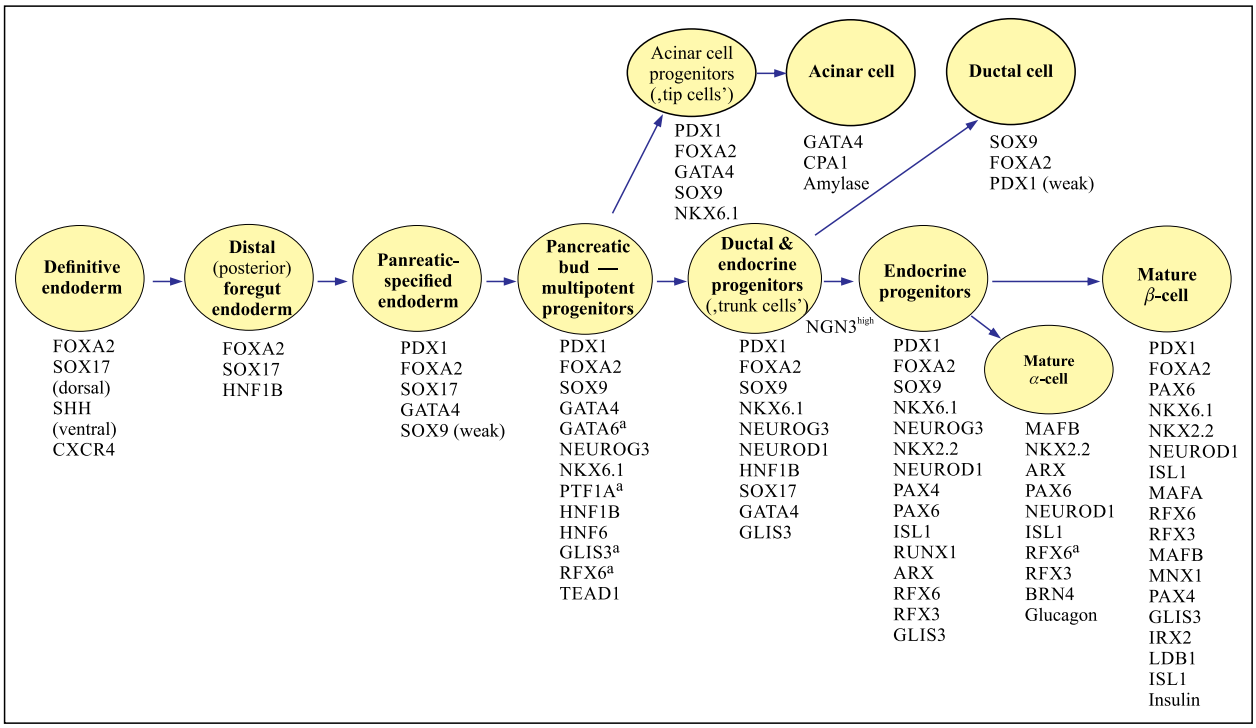


**Figure 1.** The expression of key transcription factors (TFs) at the sequential stages of the human definitive endoderm differentiation into pancreatic lineages of acinar, ductal, $\alpha$ and $\beta$ cells. The capital letters denote human TFs which were first detected at the respective phases of pancreas differentiation in mouse. [a]detected as transcripts in human embryos. Abbreviations are explained past the Abstract. Based on the data summarized by Jennings *et al.* [17], Al-Khawada *et al.* [19], Lyttle *et al.* [22], Riedel *et al.* [23], Conrad *et al.* [25], and Santosa *et al.* [41].

human pancreatic progenitor cells by the Chip-seq for PDX1 revealed a total of 8088 PDX1-bound regions that map to 5664 genes. The PDX1 target regions include important pancreatic transcription factors such as PDX1 itself, RFX6, HNF1B, and MEIS1 as well as signaling molecules and factors important for $\beta$ cell function [29]. Moreover, the application of the ChIP-seq technique for PDX1 disclosed several novel PDX1 target genes including RFX3, required in mouse for the differentiation and function of the $\beta$ cells, and the ligand of the Notch receptor DLL1, which is important for endocrine induction [29].

The *PTF1A* (pancreas transcription factor 1 subunit alpha) gene on human chromosome 10 encodes a transcription factor with a key role in early human pancreas development and cerebellar neurogenesis [26, 30]. The transcripts of the *PTF1A* have been demonstrated during the development of human pancreas; however, in contrast to mouse, the presence of PTF1A protein has not been demonstrated in humans yet, probably due to the lack of available antibodies [17]. In mouse acinar differentiation is regulated by a set of transcription factors including Ptf1a and Mist1 [31]. Ptf1a (p48) is an important bHLH factor that forms a complex with Tcf12 and Rbpjl, which allows the expression of genes for the secretory enzymes present in the mature acini [32]. It is coexpressed with Pdx1 in both dorsal and ventral pancreatic buds from E9.0 to E9.5, and later, at E12.5, Ptf1a expression is found in growing tips of the branching epithelium to eventually end up in the acinar cells [6].

Neurogenin 3 (NEUROG3, NGN3) belongs to a family of basic helix-loop-helix (bHLH) transcription factors involved in the development of central nervous system and embryonic pancreas. Neurog3 is transiently expressed in mouse [7, 33] and human pancreatic endocrine progenitor cells [15]. In mouse, there are two phases of Ngn3 expression, *i.e.* during the first and second transition, the latter peaking at E15.5 [7]. Ngn3 is required for endocrine differentiation, as demonstrated by the complete lack of all types of pancreatic endocrine cells in Neurog3-deficient mice [12]. Ngn3 initiates the differentiation of Pdx1-expressing progenitor cells and activates the expression of additional transcription factors important for the differentiation of MPCs into endocrine cell lineages [12]. In mouse single-cell lineage tracing has shown that $\alpha$ and $\beta$ cells originate from different Neurog3-expressing endocrine cells [12]. NGN3 expression during human pancreatic development starts from G8w with

a peak at the end of the first trimester [15], concomitantly with the development of islets which at G12-13w contain $\alpha$, $\beta$, $\delta$, and $\gamma$ cells [15, 20]. The NGN3 expression tightly correlates with the appearance of fetal insulin-containing $\beta$ cells; however, it becomes undetectable after G35w [34] and is not present in mature human endocrine cells [17, 22]. Interestingly, in humans NEUROG3 may not be required for the development of the endocrine pancreas since the patients with *NGN3* mutation were born with circulating C-peptide [35]. In mouse, apart from Ngn3, Pax4 and Arx transcription factors are important for the determination of endocrine cell fate [6, 36]. NGN3 is also critical for human pancreatic endocrine development since null mutations in *NGN3* cause neonatal diabetes and block $\beta$ cell differentiation from human pluripotent stem cells [37].

NKX6.1 (NK6 homeobox protein 1) and NKX2.2 transcription factors are expressed in pancreatic endoderm and endocrine precursor cells and are essential for promoting $\beta$ cell specification by controlling cell lineages between endocrine and acinar cell fate. In mouse the expression of Nkx6.1 is required for the development of the $\beta$ cell lineage from endocrine progenitors [38] so that Nkx6.1 is specifically expressed in adult $\beta$ cells. In humans the expression of NKX6.1 follows that of PDX1 and SOX9 and takes place prior to the transient activation of NGN3 that is necessary for the differentiation of $\beta$ and other islet endocrine cell types [17]. However, a recent single-cell gene expression analysis of human ESCs differentiation into $\beta$ cell lineage revealed that the activation of NKX6.1 can be initiated before or after endocrine commitment (downstream of NGN3 expression) without negative effect on the generation of $\beta$-like cells *in vitro* [39].

MAFA (v-Maf musculoaponeurotic fibrosarcoma oncogene homolog A) is a key transcription factor for fetal $\beta$ cell maturation. Nearly no MAFA is detected in human embryo until gestational week 21, and its expression gradually increases after birth, being limited at adult age to $\beta$ cells [21]. This transcription factor binds to the enhancer/promoter region of the insulin gene RIPE3b/C1-A2 and activates insulin gene expression in response to glucose [40, 41].

In addition to the transcription factors mentioned above, there are many other which participate in the control of the maintenance and expansion of multipotent pancreas progenitors and endocrine progenitors (Fig. 1). Their function and interdependency, revealed mainly in the studies of mouse pancreas development, have been extensively reviewed by Jørgensen *et al.* [6] and other authors [5, 11, 18, 19, 41–43].

Although the development of the human pancreas generally recapitulates the stages of mouse pancreas differentiation, there are some important interspecies differences which can affect the strategies for the *in vitro* $\beta$ cell differentiation, as summarized by Jennings *et al.* [17], Nair and Hebrok [44], and Balboa *et al.* [45]. For instance, the expression of the key transcription factor PDX1 necessary for the development of pancreatic progenitor cells occurs in the human endoderm later than in mouse, *i.e.* after gut closure once mesenchyme has separated notochord and aorta from dorsal foregut [15]. The expression of neurogenin 3 is biphasic during murine pancreatogenesis [7] but not in humans [15]. Other differences refer to the different timing of the expression of the transcription factors GATA4, SOX17, and NKX2.2 [6, 15]. For example, the expression of GATA4 is delayed in human pancreatic development, and SOX17, a definitive endoderm marker which exists in the presumptive human pancreatic endoderm, is lost in rodent pancreatic epithelium [15]. In the mouse, pancreatic islets are formed only close to birth (E19–21) whereas in human embryos islets appear at G12w [15]. Moreover, $\beta$ cells are the first endocrine cells detected in human pancreas [15, 20, 22] in contrast to mouse, with $\alpha$ cells forming first [33]. In mouse there are two phases of the appearance of endocrine cells during pancreatic development, referred to as primary and secondary transition, respectively. In human embryos no early pancreatic endocrine differentiation takes place so that only one wave of endocrine differentiation takes place [3, 15, 34]. Whereas in the developing human pancreas the NKX2.2 transcription factor is not expressed in multipotent pancreatic progenitor cells [15] (Fig. 1), Nkx2.2 is widely expressed in the murine pancreatic bud until E13 when its expression becomes restricted to the Ngn3-positive cells [46]. MafB expression is lost postnatally from mouse [11] but not human [23] $\beta$ cells.

In a landmark study, Jennings *et al.* [47] used laser capture, RNA amplification, and computational analysis of deep sequencing to reveal the transcriptomic programs regulating the earliest development of pancreas, liver, and biliary tree in human embryos. They found that pancreas-enriched gene expression was less conserved between human and mouse than for the liver. Apart from confirming that the dorsal pancreatic bud was enriched for components of Notch, Wnt, BMP, and FGF signaling, they discovered over 30 transcription factors which have yet not been associated with the differentiation of pancreatic endoderm [47]. Altogether, the reported differences between some stages of $\beta$ cells differentiation in mouse and human (and generally between mature

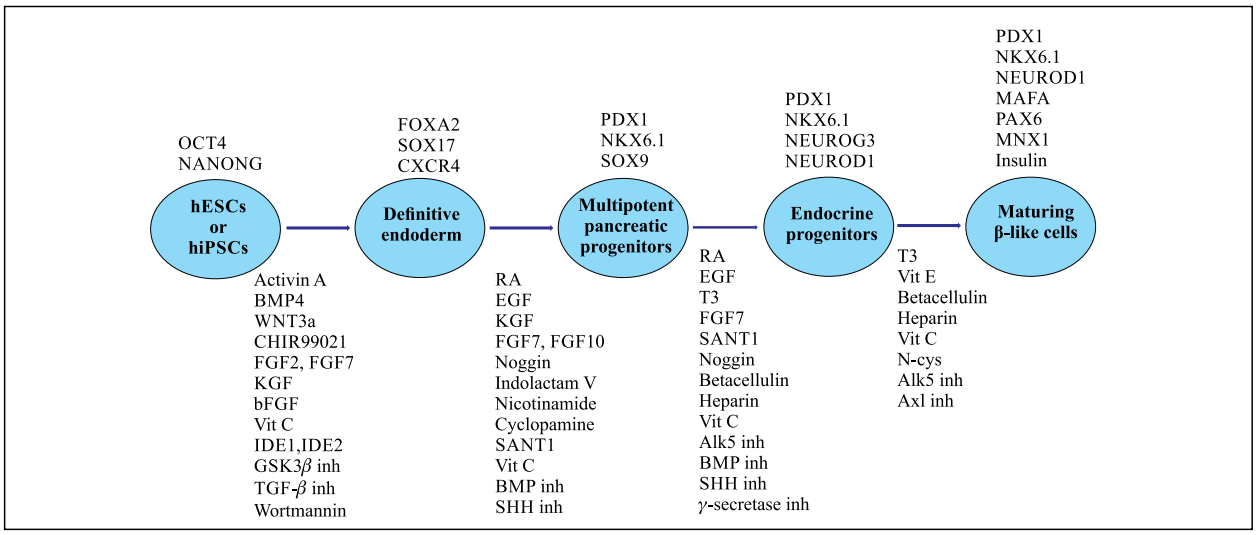


**Figure 2.** Main stages of an idealized, simplified protocol of the differentiation of hESCs or hiPSCs into $\beta$-like cells. The major markers of the differentiation stages are shown above and the growth factors and other molecules controlling the signaling pathways directing definitive endoderm cells' transformation into $\beta$ cell lineage are shown below the presented developmental stages (based on data presented in (59–64, 67–71). Abbreviations as listed on page 1 and: ALK5iII — activin receptor-like kinase 5 inhibitor II; Axl — receptor tyrosine kinase; CHIR99021 — a selective GSK3$\beta$ inhibitor; inh — inhibitor; N-cys — N-acetyl cysteine; SANT1 — Shh antagonist; T3 — triiodothyronine; wortmannin — phosphoinositide 3-kinase inhibitor.

endocrine cells in the murine and human pancreata, not mentioned in this review) suggest caution when relating findings from mice to humans. It is expected that further development of computational algorithms will allow using the transcriptomic and proteomic data derived from the studies of human embryos to facilitate stem cell research and clinical interpretation without inter-species extrapolation.

The elucidation of the chronology and hierarchy of the signaling events during mouse and human pancreas development, apart from its pure scientific importance, together with the breakthrough technologies of human embryonic stem cells' differentiation and human induced-pluripotent stem cells made it possible to generate large numbers of multipotent pancreatic progenitor cells that could be further differentiated *in vitro*, and after transplantation into immunocompromised diabetic mice develop and mature into insulin-secreting $\beta$-like cells, possibly also in humans with diabetes.

## *In vitro* recapitulation of the developmental stages of pancreas enables the reprogramming of hESCs or human iPSCs into insulin-secreting cells

The progress in the understanding of the molecular mechanisms of pancreas development, together with the advent of techniques of human ESCs differentiation [48] and generation of mouse [49] and human [50, 51] induced-pluripotent stem cells made it possible to produce *in vitro* $\beta$-like cells with the aim of replacing dysfunctional or lacking $\beta$ cells in patients with diabetes. Both types of pluripotent stem cells express unlimited proliferation capacity *in vitro* and may differentiate under appropriate culture conditions into virtually any cell type. Thus, they offer new, fascinating possibilities of future treatment of patients with diabetes by the transplantation of *in vitro* generated insulin-secreting cells. Generally, the strategies for the generation of functional $\beta$-like cells are based on mimicking *in vitro* the developmental stages of *in vivo* pancreas and $\beta$ cell development by the exposition of the sequentially cultured differentiating hESCs or human iPSCs (hiPSCs) to empirically determined cocktails of various key growth factors and small molecules which temporally activate transcription factor cascades and signaling pathways specific for the phases of definitive endoderm, pancreatic endoderm, pancreatic progenitors, endocrine progenitors, and finally of $\beta$ cell lineage differentiation (Fig. 2).

Currently, two basic strategies have been pursued for the production of $\beta$-like cells for cellular replacement therapy for patients with diabetes: (i) *in vitro* differentiation of hESCs or hiPSCs, and (ii) *in vitro* transdifferentiation of differentiated somatic cells, of-

ten, but not exclusively, of endodermal origin, without reverting to the pluripotent stage through gene manipulation [52]. The second option will not be described in this short review. However, it has to be noted that the ultimate target of both approaches is the effective functioning in patients with diabetes of insulin-secreting cells able to produce adequate amounts of insulin in response to the rapid changes of blood glucose levels.

Human embryonic stem cells obtained from the inner cell mass of human blastocysts [48] have been investigated extensively as pluripotent stem cells for the *in vitro* generation of many cell types. Scientists from the laboratory of CyThera (later Novocell, Inc., and currently ViaCyte, Inc.) described a method of effective hESC differentiation into definitive endoderm (DE) cells [53]. A year later they developed the first highly replicable multi-step protocol of the differentiation of the DE cells into pancreatic endocrine cells, which was based on mimicking pancreas development through the stages of primitive gut tube, posterior foregut, pancreatic endoderm and endocrine progenitor cells. They obtained polyhormonal cells that expressed insulin and other pancreatic hormones; however, these cells did not respond to increased glucose levels by enhanced insulin secretion [54]. After improving the *in vitro* differentiation protocol, the same group showed that after transplantation of hESC-derived immature pancreatic endoderm cells (pancreatic progenitors expressing both PDX1 and NKX6.1 transcription factors) into immunodeficient mice with experimentally-induced diabetes, normoglycemia was restored after 3–4 months, which suggested that this time was needed for the *in vivo* differentiation and maturation of these cells into glucose-responsive insulin-secreting cells [55]. These early protocols of hESC differentiation presented low efficiency, lasted for many weeks, and resulted in a heterogeneous population of insulin-immunoreactive cells that were often polyhormonal [54–56]. After improving many stages of hESC differentiation into insulin-secreting cells, a few years ago the ViaCyte started the first clinical phase I/II trial involving hESCs-derived immature pancreatic progenitor cells co-expressing PDX1 and NKX6.1 (PEC-01 cells) in patients with T1D (ClinicalTrials.gov identifier: NCT02239354, trial VX-1). These cells were placed in immunoprotective capsules and implanted under the skin of the patients. Initial data from clinical evaluation of the safety, long-term tolerability and efficacy of this system is expected to be published in the middle of the year 2019. Two years ago ViaCyte initiated two other clinical trials to test a new macroencapsulation device ("open Encaptra Device") in which direct vascularization of the PEC-01 cells can take place (ClinicalTrials.gov identifiers: NCT03162926 and NCT03163511). Although the use of the differentiated ESCs bears the risk of teratoma formation as found in diabetic mice [57], so far cancers have not been reported in the T1D patients who participate in the ViaCyte trials (www.viacyte.com). In January 2019, the Center for Beta Cell Therapy in Diabetes and ViaCyte announced the start of the European clinical trial of PEC-Direct known also as VC-2, an encapsulated pancreatic progenitor cell product designed to replace lost insulin-producing $\beta$ cells in patients with T1D (www.betacelltherapy.org).

The technique of de-differentiation of somatic cells by transcription factors'-based somatic cell reprogramming was first demonstrated for mouse adult fibroblasts by Takahashi and Yamanaka [49] who used retroviral transduction with Oct3/4, Sox2, c-Myc, and Klf4, and soon afterwards was used for the reprogramming of human dermal fibroblasts by retroviral transduction with POUF51 (OCT-4), SOX-2, KLF4, and MYC [50]. The lentiviral transduction of human fetal fibroblasts with OCT-4, SOX-2, NANONG, and LIN28 was sufficient for the generation of human induced pluripotent stem cells that had normal karyotypes, expressed telomerase, and maintained the developmental potential to differentiate into advanced derivatives of all three primary germ layers [51]. The use of hiPSCs which exhibit the morphology and growth properties of ESCs obviates the need for human embryos to obtain ESCs for *in vitro* differentiation programs into almost all types of somatic cells. Similarly to the differentiation of hESCs, the *in vitro* generation of $\beta$-like cells from hiPSCs is based on the sequential modulation of multiple signaling pathways controlling pancreas development, which has been best characterized in mouse [11], however, only partially in humans [15–17]. Substantial progress in the *in vitro* production of large numbers of $\beta$ cell progenitors was achieved by the introduction of improved 'second generation' protocols for differentiation of human ESCs [58] or human iPSCs [59, 60] into $\beta$-like cells. The protocol described by Rezania *et al.* [58] comprised 7 stages: 1. hESCs or hiPSCs, 2. Definitive endoderm, 3. Primitive gut tube, 4. Posterior foregut, 5. Multipotent pancreatic progenitors, 6. Endocrine progenitors, 7. Immature endocrine cells, 8. $\beta$-like cells. These *in vitro* differentiation steps are promoted and controlled by various empirically-defined sets of factors known to affect many signaling pathways of the respective stages of the developing pancreas in mouse and humans [11, 17–19, 24, 26, 41–45]. There have been numerous modifications of the differentiation protocols of hESCs or hiPSCs

into $\beta$-like cells aimed at the production of large populations of relatively pure multipotent pancreatic progenitors (enriched in PDX1 and NKX6.1 cells) which can be further induced into monohormonal, insulin-secreting and glucose-responsive cells. Paglucia *et al.* [59] used large-scale floating cell culture system for the differentiation of hESCs and hiPSCs by the use of a combination of 11 small molecules to obtain $\beta$-like cells through the stages of definitive endoderm, posterior gut tube, and early pancreatic progenitor cells. After 4–5 weeks, with efficiency higher than 30%, they obtained glucose-responsive, monohormonal insulin-producing cells that showed typical ultrastructure of $\beta$ cells and co-expressed key $\beta$ cell markers, including high mRNA levels of the transcription factor MAFA. Two weeks after the transplantation of these partially differentiated cells into immunodeficient diabetic NRG-Akita mice, a model of diabetes [61], normalization of blood glucose level was observed [59]. Ma and Zhu [62] have graphically summarized three improved protocols [59–61] for the differentiation of hESCs/hiPSCs into functional pancreatic $\beta$-like cells. More recently, Yabe *et al.* [63] used a six-stage protocol for the differentiation of human iPSCs to pancreatic $\beta$ cells using defined culture media without feeders or serum. They showed that induction of definitive endoderm by a selective glycogen synthase-kinase-3$\beta$ inhibitor and spheroid formation at the final stage of differentiation are important for the generation of functional $\beta$-like cells. After the transplantation of these cells under the kidney capsule of streptozotocin-induced diabetic non-obese immunocompromised (NOD/SCID) mice, blood glucose levels gradually decreased over the next 4 weeks; however, not in all transplanted animals [63]. Memon *et al.* [64] found that dissociation of densely formed definitive endoderm cells obtained from human iPSCs and re-plating them at low density, followed by a longer period of retinoic acid (RA) and FGF10 signaling resulted in a high yield of PDX1+/NKX6.1+ pancreatic progenitors. Recently, a novel protocol for rapid and footprint-free differentiation of hESCs to endocrine cells by the lipofection technique was described by Ida *et al.* [65]. The authors introduced synthetic mRNAs (synRNAs) encoding transcription factors PDX1 and NKX6.1 by the lipofection reagent into hESCs and demonstrated that pancreatic endocrine hormones were highly expressed in cells transfected with synRNA-PDX1 and synRNA-NKX6.1 at day 13, as shown by immunohistochemical staining of insulin, glucagon, and somatostatin in various populations of the obtained cells. The hESCs were able to differentiate into pancreatic endoderm cells within 3 days, and within 7–9 days into multipotent pancreatic progenitors [65]. Since growth factors are costly components of the culture media necessary for *in vitro* cell differentiation, Kondo *et al.* [66], after screening of 1250 small molecules, found that the addition of sodium cromoglicate facilitated the differentiation of endocrine precursors from multiple hiPSC/hESC lines and substantially increased the induction rate of insulin-positive cells.

Many other groups have modified culture conditions and sets of small molecules used at the sequential stages to shorten the time of the *in vitro* differentiation of hESCs or hiPSCs into insulin-secreting $\beta$-like cells and to increase the efficiency of the procedures. The various chemical modulators of the sequential *in vitro* differentiation stages have been recently characterized [67–71].

Figure 2 shows the main stages of an idealized protocol for the differentiation of hESCs or hiPSCs into $\beta$-like cells and presents the key growth factors and other molecules that affect multiple signaling pathways at each differentiation step. These *in vitro* differentiation stages mimic the developmental events that take place during murine or human pancreatogenesis and $\beta$ cell lineage development. Thus, the first stage of transforming hESCs or hiPSCs into definitive endoderm is based on mimicking Nodal and Wnt signaling pathways by activin A as well as IDE1 and IDE2, small molecules that act synergistically with activin A [72]. High concentrations of activin A mimic the proendodermal role of Nodal during *in vivo* gastrulation, whereas Wnt3a ligand, involved in endodermal patterning in mammals, was later replaced by CHIR99021 shown to be more effective [73] for the induction of definitive endoderm marked by the expression of FOXA2, SOX17 and CXCR4 (Fig. 2). The next step (for simplicity we omit here the intermediary stages of primitive gut tube and posterior foregut, as described in the original protocol by Rezania *et al.* [58]) is the formation of multipotent pancreatic progenitors (pancreatic endoderm) that is characterized by the expression of the key transcription factors PDX1 and NKX6.1. The differentiation media contain retinoic acid (RA), which activates HOX genes during *in vivo* pancreatic and liver endoderm formation, cyclopamine and SANT1 (which inhibit Shh signaling and promote pancreatic lineage development), fibroblast growth factors such as FGF7 and FGF10 (in mouse FGF10 promotes the expansion of the Pdx1-expressing endodermal cells), and Noggin, an inhibitor of BMP signaling that suppresses hepatic lineage differentiation. Moreover, activators of the protein kinase C (PKC), such as epidermal growth

factor (EGF) with nicotinamide (a poly(A-ribose) inhibitor), EGF with keratinocyte growth factor (KGF) or indolactam V (screened from over 4,000 chemicals (74)), and inhibitors of the BMP and Shh signaling (Shh inhibits the development of pancreatic endoderm in mouse) are also added to the culture media to increase the population of multipotent pancreatic progenitor cells (Fig. 2). The next stage, the generation of endocrine progenitors, is achieved in the presence of RA, EGF (required for branching morphogenesis during development of endocrine lineage in mouse pancreas [11]), triiodothyronine (T3, increases expression of neurogenin 3 necessary for $\beta$ cell differentiation and maturation), betacellulin (EGF receptor ligand that maintains NKX6.1 expression [59]), heparin, vitamin C (prevents the formation of polyhormonal cells [58]), BMP receptor inhibitor (Alk5i II, activin receptor-like kinase 5 inhibitor II), and $\gamma$-secretase inhibitor (suppresses Notch signaling). The differentiation of the endocrine progenitors into $\beta$-like cells is promoted by T3, vitamin E, betacellulin, heparin, Alk5 inhibitor, as well as Axl receptor tyrosine kinase (Axl) inhibitor and N-acetyl cysteine, which induce the expression of MAFA [58, 59] (Fig. 2).

These shortly characterized multi-step protocols of the *in vitro* differentiation of hESCs or hiPSCs into $\beta$-like cells are technically complicated, costly, take usually a few weeks, and often suffer from low differentiation efficiency, which is partially caused by the genetic variability of different hESC and hiPSC lines. To shorten the long differentiation procedures, Trott *et al.* [75] developed culture conditions by using feeder-based platform (mouse embryonic fibroblasts) that support long-term self-renewal of human multipotent pancreatic progenitors which express key pancreatic transcription factors, including PDX1 and SOX9, and exhibit transcriptomes closely related to their *in vivo* counterparts. These cells can be differentiated into glucagon- and insulin-expressing cells *in vitro* and *in vivo*. This type of approach offers a convenient alternative to pluripotent cells as a source of adult cell types for diabetes treatment and modeling of the development of human pancreas.

Southard *et al.* [76] created a pluripotent cell line derived from human primary pancreatic tissue by reprogramming the cells (without knowledge of their specificity) with non-integrating vectors, and generated definitive endoderm after a four-day differentiation protocol. The selected SR1423 cell line was differentiated according to a simplified protocol to generate after 4 weeks populations with more than 60% of insulin-expressing cells that secreted insulin in response to glucose and reversed diabetes in rodents. The authors suggest that after banking following the guidelines of Good Manufacturing Practice, the SR1423 is a candidate cell line for the production of insulin-secreting cells useful for the treatment of insulin-dependent diabetes.

Since the protocols of hESC or hiPSCs differentiation into $\beta$ cell lineage mimic the *in vivo* endocrine pancreas development, the expression of transcriptions factors or surface markers at the corresponding *in vitro* stages has been used to validate the applied protocols. Fluorescence-activated cell sorting (FACS) with specific cell surface markers has been used for the isolation and further characterization of the human fetal pancreatic cells as well as for the characterization of *in vitro* differentiating hESCs/hiPSCs cells. The examples of surface markers used for FACS-sorting of pancreatic progenitor cells include CD142 (tissue factor, a marker of pancreatic endodermal cells that also labels additional cell types) [77], CD24 (a marker for PDX1-expressing pancreatic progenitors derived from hESCs) [78], and GP2. GP2, a glycoprotein present in pancreatic secretory granules, expressed together with the key transcription factors NKX6.1 and PTF1$\alpha$, was shown to be a cell surface marker of human multipotent pancreatic progenitors [79–80]. Ramond *et al.* [81] investigated human fetal pancreatic epithelial cells by FACS analysis using a combination of the cell surface markers GP2, ECAD (also known as CDH1, *i.e.* cadherin 1), CD142, and SUSD2 (Sushi domain containing 2, a marker used to enrich NEUROG3-positive cell population from hPSC-derived pancreatic cells and the human fetal pancreas [82]). They identified distinct endocrine populations at different stages of their development (pancreatic progenitors, endocrine progenitors, and endocrine cells) and showed that a subset of the GP2-positive cells undergoes endocrine differentiation by down-regulating GP2 and CD142 and up-regulating the expression of NEUROG3, a marker of endocrine lineage differentiation [81]. The same group improved the purity of the cell populations isolated from three human fetal pancreata at G9w by applying an additional surface marker, CD133 (prominin 1, often expressed on adult stem cells), to exclude ductal cells, along with using granularity to distinguish endocrine cells [83]. The transcriptional profiling of the isolated cell populations at the single-cell level using qPCR showed that, at a single time point, different steps of the endocrine differentiation can be identified. Moreover, this approach enabled to benchmark the pancreatic cell types produced *in vitro* from one hESCs and two hiPSCs cell lines using an established endocrine-biased differentiation protocol [83]. Fur-

ther studies of single-cell transcriptomes of mouse and human progenitors at different steps of pancreas development will allow to capture intermediate stages of differentiation, increase the efficacy of the *in vitro* generation of β cell progenitors, and discover factors that will improve the functionality of the β-like cells.

## Validation of the β-like cells generated *in vitro*

Although the β-like cells show the morphological and some of the functional characteristics of the *bona fide* adult β cells, they must be regarded as immature β cells. The comparison of single cell transcriptomes and proteomes of β-like cells generated from hiPSCs with those of mature β cells revealed the lack of some molecules present in the *bona fide* β cells [84]. Numerous tests have been applied to characterize the phenotype of the *in vitro* generated β-like cells. They include the expression of specific surface molecules to perform FACS sorting, immunohistochemistry to detect insulin or C peptide, marker gene analysis by real-time PCR, the presence of potassium channels Kir6.1 and Kir6.2 and the ATP-binding cassette channel Sur1 which are necessary for insulin secretion [85]. Coupled to GSIS (glucose-stimulated insulin secretion) assessment, the presence of these proteins should be used in order to establish optimal β-like cells' response to glucose. The functional assays include testing of the extent of glucose-stimulated insulin secretion in terms of its amount and stimulation index (*i.e.* the ratio of insulin secretion levels between low- and high-glucose concentrations), fluctuations of $Ca^{2+}$ intracellular levels, or secretion of C-peptide. Initially, the hESC-derived β-like cells presented suppressed glucose-stimulated insulin secretion [56], and in response to a rapid increase of glucose concentration they did not reproduce the high amplitudes of fast changes of $Ca^{2+}$ concentrations that are a hallmark of the mature β cells isolated from human pancreatic islets [84, 85].

## Concluding remarks

The ultimate target of *in vitro* produced β-like cells is their phenotypic and functional similarity to the authentic β cells of human pancreas. Despite the continuous improvements in the differentiation protocols of human ESCs or hIPSCs for scalable *in vitro* generation of β-like cells, the time is not yet ready for their use as an alternative diabetes treatment. It has been shown that there are differences between different hESCs and hiESCs in their transcriptomic profiles and efficacy of reprogramming into β-like cell progenitors [86]. Moreover, the β-like cells rarely present pure populations since they are often contaminated by other endocrine cell types and are sometimes polyhormonal. Thus, reproducibility and standardization of the *in vitro* differentiation processes present essential requirements that have to be clearly defined for the acceptance of the β-like cells for future therapies by health regulatory authorities. There are important ethical concerns associated with the use of embryonic cells from destroyed human embryos. Also the use of human induced pluripotent stem cell lines carries the risk of transmitting possible disease hereditary traits from a donor.

It has been demonstrated that *in vitro* generation of β-like cells is possible also with iPSCs derived from patients with T1D and T2D [87–89] and maturity-onset diabetes of the young [90–92]. It is hoped that generation of hiPSCs derived *e.g.* from fibroblasts of patients with diabetes [93] will improve our understanding of the underlying genetic abnormalities, provide targets for high-throughput screening of thousands of molecules as possible therapeutics, and offer personalized treatment by autologous transplantation, since the allogenic transplantation of hPSC-derived β-like cells is likely to induce an immune response. The advancements in the engineering of encapsulation devices that allow for the contact of the transplanted cells with the invading blood vessels but protect them from contact with the immune cells provide hope that these obstacles could be overcome in the near future [94–97]. Obviously, we need more clinically relevant information from the ongoing clinical trials of the diabetes treatment with hESC- or hiPSC-derived β-like cells regarding the functionality of the transplanted cells and immune reactivity of the recipients. Since all these important problems are also relevant for the β-like cells obtained from *in vitro* direct transdifferentiation of differentiated somatic cells, they will be discussed in our next review paper.

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# The impact of sedentary work on sperm nuclear DNA integrity

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**Abstract**

**Introduction.** Contemporary professional jobs that often enforce a sedentary lifestyle and are frequently associated with testicular overheat, deserve special attention with respect to male fertility potential. Interestingly, the harmful effect of testicular heat stress on sperm characteristics including nuclear DNA integrity was well characterized; however, the influence of sedentary work on sperm chromatin has not yet been documented. Therefore, our research was designed to examine the potential effects of sedentary work not only on conventional semen features but also on sperm nuclear DNA status.

**Materials and methods.** The study was carried out on ejaculated sperm cells obtained from men who spent ≥ 50% of their time at work (≥ 17.5 h per week) in a sedentary position (n = 152) and from men who spent < 50% of their time at work in a sedentary position (n = 102). Standard semen characteristics were assessed according to the WHO 2010 recommendations, while sperm nuclear DNA fragmentation (SDF) was evaluated using the Halosperm test.

**Results.** There were no significant differences in the standard semen parameters between the study groups. The groups differed only in SDF parameter. The men who spent at least 50% of their work time in a sedentary position had a higher proportion of SDF than the men who spent < 50% of their time at work in a sedentary position (median value 21.00% *vs.* 16.50%, respectively). The incidence of low SDF levels (related to 0–15% sperm cells with abnormal DNA dispersion) was significantly lower (27.63% *vs.* 45.10%), the percentage of men with high SDF levels (related to > 30%) was significantly higher (30.92% *vs.* 16.67%) in group of men who spent at least 50% of their work time in a sedentary positon. Furthermore, these men were more than twice as likely to have not a low SDF level (OR: 0.4648) and had more than twice the risk of having a high SDF level (OR: 2.2381) than the men in less sedentary occupations.

**Conclusions.** Despite lack of association between sedentary work and conventional semen characteristics our study revealed detrimental effect of seated work on sperm nuclear DNA integrity. A sedentary job doubled the risk of high levels of sperm DNA damage. The pathomechanism could be related to testicular heat stress resulting in sperm chromatin remodelling failure during spermiogenesis. Therefore, it seems reasonable to simultaneously carry out routine seminological analyses and tests assessing sperm chromatin status while diagnosing male infertility. *(Folia Histochemica et Cytobiologica 2019, Vol. 57, No. 1, 15–22)*

**Key words:** sperm parameters; DNA fragmentation; sedentary work; male reproductive health

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## Introduction

Infertility has become a global disorder affecting up to 20% of couples trying to conceive. Infertility is not only a medical challenge but also a sociological and economic issue [1–3]. Couples affected by this problem struggle with stress, depression, and family crisis, even leading to divorce. Moreover, treatment is very expensive and not universally affordable [1–4]. It is estimated that male factors (coexisting with female factors) contribute to infertility in up to half or even 70% of infertility cases (20–43% in Africa, 37% in Asia, 40% in Oceania, 50% in North America, 50–56%, in Europe, 52% in South America, 70% in Middle-East). One-third of these cases can be caused by male factors alone [1–3, 5, 6]. Male factors (*e.g.*, congenital and acquired urogenital defects, urogenital infections, genetic, hormonal and immunological disorders, cancers, systematic diseases, age, lifestyle) can affect fertilization, embryo gene expression and development. They may also be responsible for idiopathic pregnancy loss as well as autosomal dominant diseases and neurobehavioural disorders in offspring, especially in cases of advanced paternal age [7–12].

It should be emphasized that unhealthy lifestyle of men (*e.g.*, drug use, smoking, alcohol consumption, obesity, psychological stress, environmental pollution) are very important and simultaneously modifiable factors affecting male reproductive ability [13–17]. Some reports revealed that contemporary professional jobs that are often associated with a sedentary lifestyle may contribute to declining semen characteristics (*e.g.*, number of sperm cells, sperm motility, morphology) due to testicular heat stress [18–21]. Importantly, a physiological temperature of human testes between 32°C and 35°C (the optimal temperature is 1–2°C below the body core temperature) is needed for the normal course of spermatogenesis associated with correct essential gene expression. Therefore, the differentiation and maturation of spermatocytes and spermatids, germ cells of seminiferous epithelium, are temperature-dependent processes. However, a seated position leads to testes overheating due to poor air circulation in the groin area and impaired blood flow. The increase in the scrotal temperature may trigger pathological changes in both seminiferous epithelium (Sertoli cells and germinal cells) and endocrine cells located in testicular interstitial tissue, *i.e.* Leydig cells. Therefore, spermatogenic arrest leading to a decrease in sperm concentration or even testicular atrophy may appear [22, 23]. Likewise, heat stress induces damage of mitochondria, dilatation of the smooth endoplasmic reticulum in germinal cells and increases the distance between spermatids and Sertoli cells. In the latter case, premature exfoliation of immature spermatids is often associated with their apoptosis [22–24]. Furthermore, DNA damage (defects in chromosomes' synapsis, DNA strand breaks, suppression of DNA repair) is observed in germinal cells, particularly in pachytene and diplotene spermatocytes. The DNA damage may be caused not only by the direct action of heat stress but also by the pathological heat-dependent generation of reactive oxygen species (ROS) [22–24].

It should be highlighted that the harmful effect of testicular heat stress on sperm nuclear DNA integrity was well elaborated but the influence of sedentary work on sperm chromatin has not yet been documented. Therefore, our study was designed to examine the potential effects of sedentary work not only on conventional semen features but also on sperm nuclear DNA status.

## Materials and methods

**Subjects.** The study was carried out on ejaculated sperm cells obtained from general population of men (n = 254) attending the Andrology Laboratory of the Department of Histology and Developmental Biology (Pomeranian Medical University in Szczecin, Poland). We designed a questionnaire to record personal characteristics and medical history. Men also reported the average time *per* week spent in a sedentary position at work. Subjects who worked at least 35 h *per* week were considered for seminological analyses. Based on Støy *et al.* [25], the subjects were divided into two groups: group 1 comprised men who spent ≥ 50% of time at work in a sedentary position, *i.e.*, a minimum of 17.5 h per week (n = 152, median age = 31.50 years), whereas group 2 comprised men who spent < 50% of time at work in a sedentary position (n = 102, median age = 31.00 years). In both groups, the exclusion criteria included the following: working time less than 35 h *per* week, a clinical picture suggestive of obstructive azoospermia, a history of testicular torsion, varicocele, maldescent of testis, cryptorchidism, injury or cancer and co-existing systemic disease. The ethics committee of the Pomeranian Medical University, Szczecin, Poland approved the study protocol (ethical authorization number: KB-0012/21/18).

**Conventional semen analyses.** The semen samples were obtained by masturbation after 2–7 days of sexual abstinence. The standard semen characteristics were evaluated according to the WHO 2010 recommendations [26]. Sperm concentration was estimated in an improved Neubauer hemocytometer (Heinz Hernez Medizinalbedarf GmbH, Hamburg, Germany), whereas sperm motility (total and progressive motility), vitality (live sperm cells: eosin-negative or hypo-osmotic-reactive sperm cells – HOS test) and morphology in a bright light microscope (CX 31, Olympus Optical Co., Ltd., Tokyo, Japan). Papanicolaou-stained

spermatozoa was used for sperm morphology and teratozoospermia index (TZI) assessment. Concentration of leukocytes in the semen samples (peroxidase-positive cells) was calculated using the Endtz test (LeucoScreen kit, FertiPro N.V., Beernem, Belgium).

A normozoospermia (positive results of the basic seminological examination) was considered according to the following criteria: sperm concentration ≥ 15 mln/mL, total number of sperm cells ≥ 39 mln, sperm progressive motility ≥ 32% and morphology ≥ 4%. Furthermore, the teratozoospermia index, vitality and concentration of peroxidase-positive cells (leukocytes) were evaluated (Table 1). In the group of 152 men who spent ≥ 50% of time at work in a sedentary position, the following seminological categories were distinguished: normozoospermia (n = 54), asthenozoospermia (abnormal sperm motility, n = 2), oligozoospermia (abnormal number of sperm cells, n = 3), teratozoospermia (abnormal sperm morphology n = 40), asthenoteratozoospermia (abnormal sperm motility and morphology, n = 17), oligoteratozoospermia (abnormal sperm number and morphology, n = 15) and oligoasthenoteratozoospermia (abnormal sperm number, motility and morphology, n = 21). For the group of 102 men who spent < 50% of time at work in a sedentary position, the following categories were identified: normozoospermia (n = 42), asthenozoospermia (n = 1), oligozoospermia (n = 2), teratozoospermia (n = 28), asthenoteratozoospermia (n = 10), oligoteratozoospermia (n = 9) and oligoasthenoteratozoospermia (n = 10).

**Sperm chromatin dispersion (SCD) test (Halosperm test).** The SCD test was used to assess sperm nuclear DNA fragmentation (SDF). The evaluation of DNA fragment dispersion after denaturation was carried out using a Halosperm G2 kit (Halotech DNA, Madrid, Spain) following the manufacturer's guidelines. Semen samples were diluted with PBS (phosphate buffered saline, pH = 7.4) to adjust the concentration to no more than 20 mln/mL. Agarose gel from the kit was incubated for 5 min in hot water at 95°C to ensure complete melting and then was incubated at 37°C. Fifty microliters of warm agarose (37°C) was added and mixed with 25 $\mu$L of semen in an Eppendorf tube. The mixtures (10 $\mu$L) were placed on a super-coated slide and covered with a coverslip. The smears were kept for 5 minutes at 4°C in a fridge to solidify the agarose. Thereafter, the coverslips were carefully removed, and the reaction area was fully immersed in a denaturation solution for 7 min. The smears were drained by tilting the slides. Afterwards, the reaction area was fully immersed in the lysing solution for 20 min. Then, the smears were placed in distilled water for 5 min and dehydrated by flooding with 70% ethanol and then 100% ethanol (each for 2 min). After drying, the slides were stained with an eosin staining solution and thiazine staining solution (each for 7 min).

**Sperm DNA fragmentation scoring.** The smears were evaluated under a bright light microscope at 1000× magnification (CX 31 Olympus microscope). A minimum of 300 spermatozoa *per* sample were counted. Sperm cells without SDF can produce the characteristic halo of dispersed DNA loops (large halo: halo width similar to or higher than the diameter of the sperm head; medium halo: halo width > 1/3 the diameter of the sperm head), while spermatozoa with damaged DNA fail to form a halo of dispersed DNA loops (small halo: halo width ≤ 1/3 the diameter of the sperm head; sperm cells without a halo or with degraded DNA: spermatozoa with no halo or irregularly, weakly stained sperm head) (Fig. 1). The results are presented as the total number of spermatozoa with small or no halo, *i.e.*, degraded, divided by the total number of assessed sperm cells and multiplied by 100% [16]. The levels of sperm chromatin damage were estimated based on the following criteria: 0–15% SDF (low level of sperm cells with fragmented DNA, high fertility potential), 16–30% SDF (moderate level, moderate fertility potential) and > 30% SDF (high level, low fertility potential) [27–30].

**Statistical analysis.** The statistical analyses were performed using Statistica version 13.3 (StatSoft, Poland) and MedCalc version 15.2.2 (MedCalc Software, Ostend, Belgium) software with significance set at $p < 0.05$. The quantitative variables are expressed as the mean ± standard deviation (SD) and median (range), while qualitative data are reported as percentages. The conformity of numerical variables with the normal distribution was examined using the Shapiro-Wilk test. The nonparametric Mann-Whitney U test was used to compare data from two independent groups. A chi-square test was used to compare the categorical data. The odds ratios (ORs) for SDF levels (their 95% confidence intervals and p value) to define the relative risk in predicting the level of SDF in men who spent ≥ 50% of time at work in a sedentary position with respect to men who spent < 50% were calculated using the method of Altman [31].

## Results

The compared groups did not significantly differ in body mass index (BMI) (the evaluation was carried out by medical staff), *i.e.*, kilograms of body weight divided by the square of the person's height in metres [32], age and standard semen parameters, but they did differ in SDF (Table 1). The men who spent at least 50% of their professional work time in a sedentary position (Group 1) had a higher proportion of SDF than the men who spent less than 50% of their work time in a sedentary position (Group 2); median: 21.00% *vs.* 16.50%, respectively. Moreover, the groups differed significantly in the incidence of low SDF levels (27.63% *vs.* 45.10% of subjects, respectively) and high SDF levels (30.92% *vs.* 16.67% of subjects,

**Table 1**. Descriptive statistics and comparison of body mass index (BMI), standard semen parameters and sperm DNA fragmentation (SDF) between groups of men who spent ≥ 50% of time at work in a sedentary position (Group 1) and men who spent < 50% of time at work in a sedentary position (Group 2)

| Parameter | Reference = normal values | Group 1 (n, median with range and or mean ± SD) | Group 2 (n, median with range and mean ± SD) | *P* |
|---|---|---|---|---|
| **BMI** | 18.50–24.99 kg/m$^2$* | 150<br>25.75 (19.40–37.46)<br>26.34 ± 3.24 | 100<br>25.90 (18.83–47.77)<br>26.50 ± 4.42 | 0.574469 |
| **Age (y)** | no data | 152<br>31.50 (21.00–51.00)<br>32.9 6 ± 5.67 | 102<br>31.00 (22.00–48.00)<br>32.16 ± 5.98 | 0.202004 |
| **Semen volume (mL)** | ≥ 1.50 mL** | 152<br>3.22 (1.10–11.50)<br>3.50 ± 1.55 | 102<br>3.00 (0.50–8.80)<br>3.46 ± 1.63 | 0.588980 |
| **Sperm concentration (× 10$^6$/mL)** | ≥ 15 mln/mL** | 152<br>22.55 (0.25–166.00)<br>33.22 ± 34.01 | 102<br>27.69 (0.80–283.00)<br>37.74 ± 4 0.10 | 0.157160 |
| **Total number of spermatozoa (× 10$^6$)** | ≥ 39 mln** | 152<br>73.10 (0.75–660.25)<br>104.33 ± 99.98 | 102<br>79.07 (1.60–566.00)<br>121.45 ± 118.45 | 0.307699 |
| **Morphologically normal spermatozoa (%)** | ≥ 4%** | 152<br>2.00 (0.00–13.00)<br>2.98 ± 2.88 | 102<br>3.00 (0.00–12.00)<br>3.33 ±3.02 | 0.373186 |
| **TZI** | ≤ 1.8** | 152<br>1.53 (1.20–2.14)<br>1.56 ± 0.17 | 102<br>1.50 (1.17–2.09)<br>1.52 ± 0.16 | 0.098516 |
| **Progressive motility (%)** | ≥ 32%** | 152<br>49.00 (0.00–86.00)<br>46.15 ± 21.70 | 102<br>53.50 (0.00–87.00)<br>50.25 ± 21.86 | 0.082646 |
| **Non-progressive motility (%)** | no data | 152<br>5.00 (0.00–23.00)<br>6.39 ± 4.18 | 102<br>5.00 (0.00–26.00)<br>5.95 ± 4.03 | 0.540435 |
| **Total sperm motility (%)** | ≥ 40%** | 152<br>57.00 (0.00–92.00)<br>52.55 ± 22.02 | 102<br>60.50 (0.00–91.00)<br>56.20 ± 22.04 | 0.167037 |
| **Eosin-negative spermatozoa — live cells (%)** | ≥ 58%** | 152<br>75.50 (2.00–94.00)<br>71.54 ± 16.67 | 102<br>79.00 (0.00–95.00)<br>73.40 ± 18.99 | 0.060613 |
| **HOS test-positive spermatozoa — live cells (%)** | ≥ 58%** | 145<br>73.00 (0.00–94.00)<br>69.68 ± 16.46 | 96<br>77.50 (0.00–93.00)<br>71.18 ± 19.47 | 0.066224 |
| **Peroxidase-positive cells (mln/mL)** | < 1.00 mln/mL** | 152<br>0.25 (0.00–10.00)<br>0.59 ± 1.35 | 102<br>0.25 (0.00–6.50)<br>0.50 ± 0.90 | 0.649891 |
| **SDF** | < 30%*** | 152<br>21.00 (5.00–89.00)<br>24.76 ± 15.01 | 102<br>16.50 (3.00–89.00)<br>19.99 ± 13.73 | 0.001816 |

n — number of subjects, HOS test — hypo-osmotic swelling test; SD — standard deviation, TZI — teratozoospermia index, p — significance of differences between compared groups, Mann-Whitney U test. *reference values according to WHO 2000 recommendations [32] (2000); **reference values according to WHO 2010 recommendations [26]; *** reference values according to Evenson and Wixon [42].

respectively) (Table 2). Furthermore, men in the Group 1 had significantly lower ORs for a low SDF level (OR: 0.4648) and significantly higher ORs for a high SDF (OR: 2.2381) than the men in Group 2 (Table 3).

## Discussion

In our study, we analysed relationships between seminological parameters and a sedentary work position. Therefore, considering the suggestions of

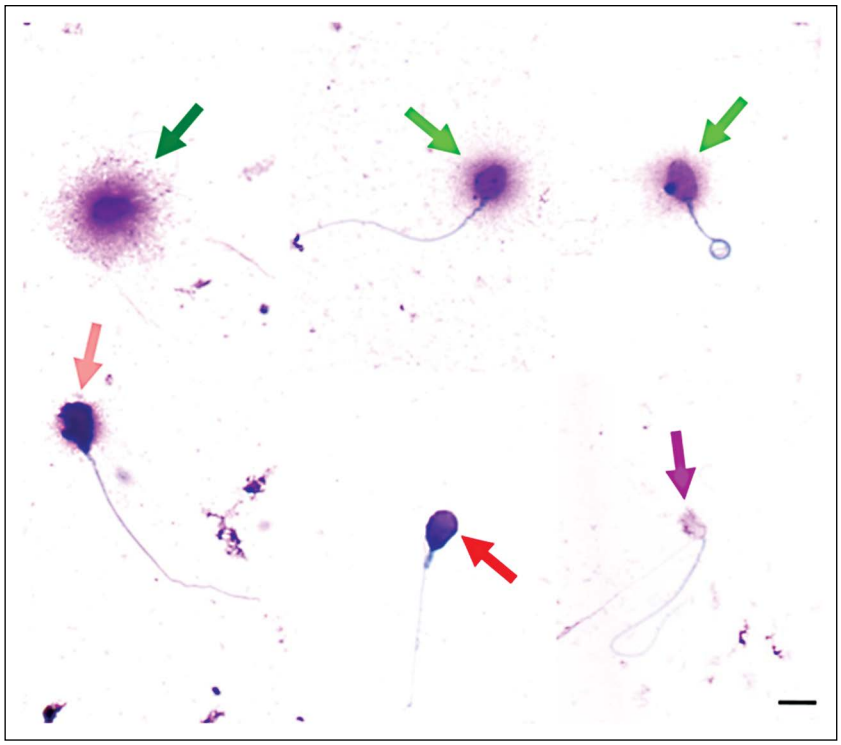

**Figure 1.** Micrographs presenting the results of the sperm chromatin dispersion test. Sperm cells without fragmented nuclear DNA: large halo (dark green arrow) and medium halo (bright green arrows). Sperm cells with fragmented nuclear DNA: small halo (rose arrow) and no halo (red arrow). Sperm cell with degraded DNA (purple arrow). Scale bar = 10 $\mu$m.

**Table 2.** Prevalence of sperm chromatin fragmentation (SDF) levels in groups of men who spent ≥ 50% of time at work in a sedentary position (Group 1) and who spent < 50% of time at work in a sedentary position (Group 2)

| Group | SDF (%) | | |
|---|---|---|---|
| | **0–15% n (%)** | **16–30% n (%)** | **> 30% n (%)** |
| Group 1 (n = 152) | 42 (27.63) | 63 (41.45) | 47 (30.92) |
| Group 2 (n = 102) | **46 (45.10)**** | 39 (38.24) | **17 (16.67)*** |

n — number of subjects; *significant difference between compared groups at p = 0.0157; **significant difference between compared groups at p = = 0.0063 (bold); Chi$^2$ test.

**Table 3.** Odds ratio (OR) of forming sperm chromatin fragmentation (SDF) levels in the group of men who spent ≥ 50% of work time in a sedentary position (Group 1) and who spent < 50% of work time in a sedentary position (Group 2)

| | **Group 1 (n = 152)** | **Group 2 (n = 102)** | **OR (95%CI)** |
|---|---|---|---|
| **SDF 0–15%** | 42 (27.63) | 46 (45.10) | **0.4648** (0.2742–0.7879)** |
| **SDF 16–30%** | 63 (41.45) | 39 (38.24) | 1.1435 (0.6843–1.9108) |
| **SDF > 30%** | 47 (30.92) | 17 (16.67) | **2.2381* (1.1991–4.1775)** |

n — number of subjects and percentage of the whole group in parentheses; *statistical significance at p = 0.0114, **statistical significance at p = = 0.0044. Test of significance, the P-value is calculated according to Sheskin [43], 95%CI — 95% confidential interval.

other authors [25], the study subjects were divided into two groups: men who spent ≥ 50% of working time in a sedentary position (≥ 17.5 h per week) and men who spent < 50% of their time at work in a sedentary position. The obtained data suggested no association between sedentary work and conventional semen characteristics. Our findings are in agreement with the data obtained by other researchers [18, 19, 25, 33, 34]. Støy *et al.* [25] suggested that sedentary work was not a risk factor for abnormal semen characteristics. Moreover, De Fleurian *et al.* [34] did not observe differences in the prevalence of normal and abnormal conventional semen parameters with respect to the number of hours spent in a sitting position. In turn, Figà-Talamanca *et al.* [19] published results that were partly consistent with our data. They com-

pared semen parameters of taxi drivers with those of healthy individuals. The authors showed a significant difference only in the proportion of normal sperm cell morphology (taxi drivers had fewer normal sperm forms). In another publication, Boggia *et al.* [18] described that men who worked in sitting positions and those who had free work positions differed only in total sperm motility. Furthermore, Magnusdottir *et al.* [33] reported that the prevalence of sedentary work was significantly lower among men with high normal sperm concentration compared to that for men with low normal sperm concentration.

It should be highlighted that we did not observe a significant difference in the standard semen parameters between men who spent ≥ 50% of their work time in a sedentary position (≥ 17.5 h per week) and men who spent < 50% of their time at work in a sedentary position. However, we found a difference in the percentage of spermatozoa with SDF, the prevalence of low and high SDF levels and the ORs for a low or high proportion of sperm cells with abnormal DNA structure. Moreover, the incidence of high DNA damage (> 30% sperm cells with SDF) was also significantly higher in this group and could be related to a low fertility potential [27–30] because a high proportion of sperm cells with DNA damage is associated with negative effects on fertilization, embryo and pregnancy rate [28, 35–37]. Therefore, we can conclude that sedentary work affects sperm DNA and doubles the risk of having a high SDF level without changing conventional semen characteristics and could lead to reduced male fertility.

It has to be noted that according to our best knowledge this is the first study about the influence of sedentary work on sperm chromatin status. It is interesting to consider which pathomechanism could be responsible for sperm DNA damage in 'sedentary' men. It has been shown that a sitting position may lead to testicular heat stress [20, 38, 39], which may provoke DNA damage [21–24, 40, 41]. As reported by Koskelo *et al.* [38], only 20 minutes of sitting in an office chair can increase the scrotal temperature by up to 3°C. Moreover, Bujan *et al.* [39] observed that the mean scrotal temperature increased in drivers after 2 hours of driving, reaching a value 1.7–2.2°C higher than the corresponding mean scrotal temperature during walking. Additionally, Hjollund *et al.* [20] showed that in periods of sedentary work, the median scrotal temperature was on average 0.7°C higher. It has been proven that a high body temperature reduces sperm DNA integrity [21, 40, 41]. Sergerie *et al.* [40] revealed that a 2-day fever of 39–40°C significantly affected sperm cell concentration, motility and vitality as well as sperm DNA integrity. The proportion of sperm with DNA fragmentation increased from 9% (before fever) to 24% and 36% (15 and 37 days after fever, respectively). In turn, in a prospective randomized clinical study, Rao *et al.* [21] showed that men undergoing warming in a 43°C bath had significantly affected sperm DNA integrity as well as reduced standard semen parameters. Moreover, a direct impact of temperature on sperm DNA fragmentation was reported by Santiso *et al.* [41], who incubated sperm cells at 37°C, 41°C and 45°C for 24 hours. The authors revealed that a higher incubation temperature was associated with a higher SDF. Based on the described data, we can speculate that an increase in the proportion of spermatozoa with abnormal DNA integrity in men who spent ≥ 50% of their work time in a sedentary position was most likely related to the testicular temperature stress.

## Conclusions

Our study suggests that an influence of sedentary work on semen fertility potential is possible but not clearly verifiable. Although this and other studies revealed a lack of association between sedentary work and standard semen characteristics, we have demonstrated the detrimental effect of sedentary work on sperm nuclear DNA integrity since the sedentary job doubled the risk of high sperm DNA damage. We can speculate that the discovered DNA damage could be related to testicular heat stress resulting in sperm chromatin remodelling failure during spermiogenesis. Therefore, it seems reasonable to simultaneously carry out routine seminological analyses and tests assessing sperm chromatin status while diagnosing male infertility.

## Conflicts of Interest

The authors declare there is no conflict of interest regarding the publication of this article.

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ORIGINAL PAPER

VIA MEDICA

# Immunohistochemical demonstration of LH/CG receptors in non-neoplastic human adrenal cortex and adrenocortical tumors

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**Abstract**
**Introduction.** Numerous data indicate that luteinizing hormone and/or chorionic gonadotropin (LH/CG) exert direct actions on the adrenal cortex and are involved in the adrenal pathology. However, the immunohistochemical studies on the expression of LH/CG receptors (LH/CGR) in the human adrenal cortex and in the adrenocortical tumors are scarce.
**Material and methods.** Paraffin sections of samples of 6 human non-neoplastic adrenal cortex and 25 adrenocortical tumors were immunostained with anti-LH/CGR polyclonal antibody.
**Results.** All zones of the human non-neoplastic adrenal cortex present a positive immunoreaction with anti-LH/CGR antibody showing the strongest reaction in cell membranes. The LH/CGR immunostaining in the vast majority of hormonally non-functioning adenomas and in all hormone-secreting adenomas does not differ from the non-neoplastic adrenal cortex. In contrast to non-neoplastic adrenal cortex and benign adenomas, in adrenocortical cancers the immunostaining with anti-LH/CGR antibody behaves differently. The immunopositive material is almost totally filling the cytoplasm of the cells but the immunopositivity of cell membranes is weak or lacking.
**Conclusions.** The data presented in our study show that the expression of LH/CGR in adrenocortical tumors is not ectopic but eutopic. The immunohistochemical examination of LH/CGR may be useful in the differentiation between benign and malignant lesions in the adrenal cortex. Moreover, the loss of membrane localization of LH/CGR in adrenocortical cancer suggests the alteration of receptors' function. *(Folia Histochemica et Cytobiologica 2019, Vol. 57, No. 1, 23–27)*

**Key words:** adrenal cortex; adrenocortical adenomas; adrenocortical cancer; luteinizing hormone/chorionic gonadotropin receptor

## Introduction

The data indicating the direct actions of gonadotropins on the adrenal gland were published as early as in the sixties of the 20th century [1–3]. The quoted authors observed the morphological changes in the adrenal cortex of the rat under the influence of exogenous administration of LH and/or FSH. Since the model of gonadectomized-hypophysectomized animals was used in the experiments, the mediation of gonadal steroids could be excluded.

In the forthcoming years the gonadotropin receptors' structure was elucidated as membrane G-protein-coupled elements possessing seven transmembrane domains [4]. The further studies showed the presence of lutropin/chorionic gonadotropins receptors (LH/CGR) in the normal human adrenal cortex [5, 6]

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**Table 1.** The source of adrenocortical samples included in the study

| Histopathological diagnosis | Clinical status | No of patients |
|---|---|---|
| Normal adrenal cortex | No alterations of the adrenal function | 3 |
| Adrenocortical hyperplasia | No alterations of the adrenal function | 3 |
| Adrenocortical adenoma | Non-functioning tumor | 11 |
| Adrenocortical adenoma | Conn's syndrome | 5 |
| Adrenocortical adenoma | Cushing's syndrome | 4 |
| Adrenocortical cancer | Malignant tumor, Cushing's syndrome | 1 |
| Adrenocortical cancer | Non-functioning malignant tumor | 4 |

in human adrenocortical aldosterone-secreting [6–8] or cortisol-secreting adenomas [9–10] and adrenocortical cancers [11]. The functional LH/CGR were also found in human adrenocortical cancer cell line H 295R cells [12]. The data on LH/CGR in adrenal tissues were collected mostly using molecular biology techniques [6–11] but no systemic immunohistochemical studies of adrenocortical tumors and non–tumoral human adrenal cortex are available.

## Material and methods

**Samples of adrenal glands**. The archival material of 25 surgically excised adrenal tumors stored in paraffin blocks was studied. The detailed presentation of material was shown in Table 1. In addition 6 non-tumoral adrenal glands were studied. Two of them were removed from patients suffering from renal cancers during the surgical excision of the affected kidney, one was excised together with pheochromocytoma tumor, and 3 were diagnosed as adrenocortical hyperplasia. The study was approved by the Ethical Committee of the Medical University of Lodz, decision RNN/335/17/KE dated 21 November 2017.

**Immunohistochemistry.** Paraffin sections were immunostained with anti-LH/CGR polyclonal antibody PA 1552 (Boster Biologicals Technologies, Pleasanton, CA, USA). This antibody binds the N-terminal part of LH/CGR (127-143 aa). The slides were incubated with antibody (1:150) for 24 h at 4°C The visualization of immunostaining was performed using Envision kit (DAKO, Glostrup, Denmark) with the use of 3,3' diaminobenzidine (DAB) as chromogen. For a positive control, a biopsy sample of the human testis was immunostained (Fig. 1A). For a negative control, the primary antibody was omitted in the immunostaining procedure.

**Statistical analysis.** Since we present only qualitative descriptions no statistical analysis was performed.

## Results

All zones of the human non-neoplastic adrenal cortex show a positive immunoreaction with anti-LH/CGR antibody. In zona glomerulosa and zona fasciculata the strongest immunoreaction is visible in cell membranes (Fig. 1B and C). On the other hand, the cytoplasmic area of adrenocortical cells is mostly empty because of the washing out of the fat deposits. A scarce residual immunostaining was also present. In zona reticularis the distribution of immunoreaction is similar but a part of cells contain the abundant immunoreactive material in their cytoplasm (Fig. 1D).

The LH/CGR immunostaining in the vast majority (10/11) of hormonally non-functioning adenomas and in all cortisol-secreting adenomas does not differ from the non-neoplastic glomerulosa and fasciculata zones (Fig. 2A and B). In one case of non-functioning adenoma the loss of immunoreaction within cell membranes and homogenous immunoreaction of cytoplasm was noticed. In aldosterone-producing adenomas (Conn's syndrome) the immunostaining of cell membranes does not differ from normal zona glomerulosa, but the density of the immunoreaction of cytoplasm is more variable (Fig. 2C).

In contrast to non-neoplastic adrenal cortex and benign adenomas, in adrenocortical cancers the immunostaining with anti-LH/CGR antibody behaves differently. The immunopositive material of variable intensity is almost totally filling the cell cytoplasm. In contrast, the immunopositivity of cell membranes is very weak or lacking except one case (Fig. 2D).

## Discussion

The expression of LH/CGR in non-neoplastic adrenal cortex and in certain adrenal tumors is well established in many studies conducted with molecular

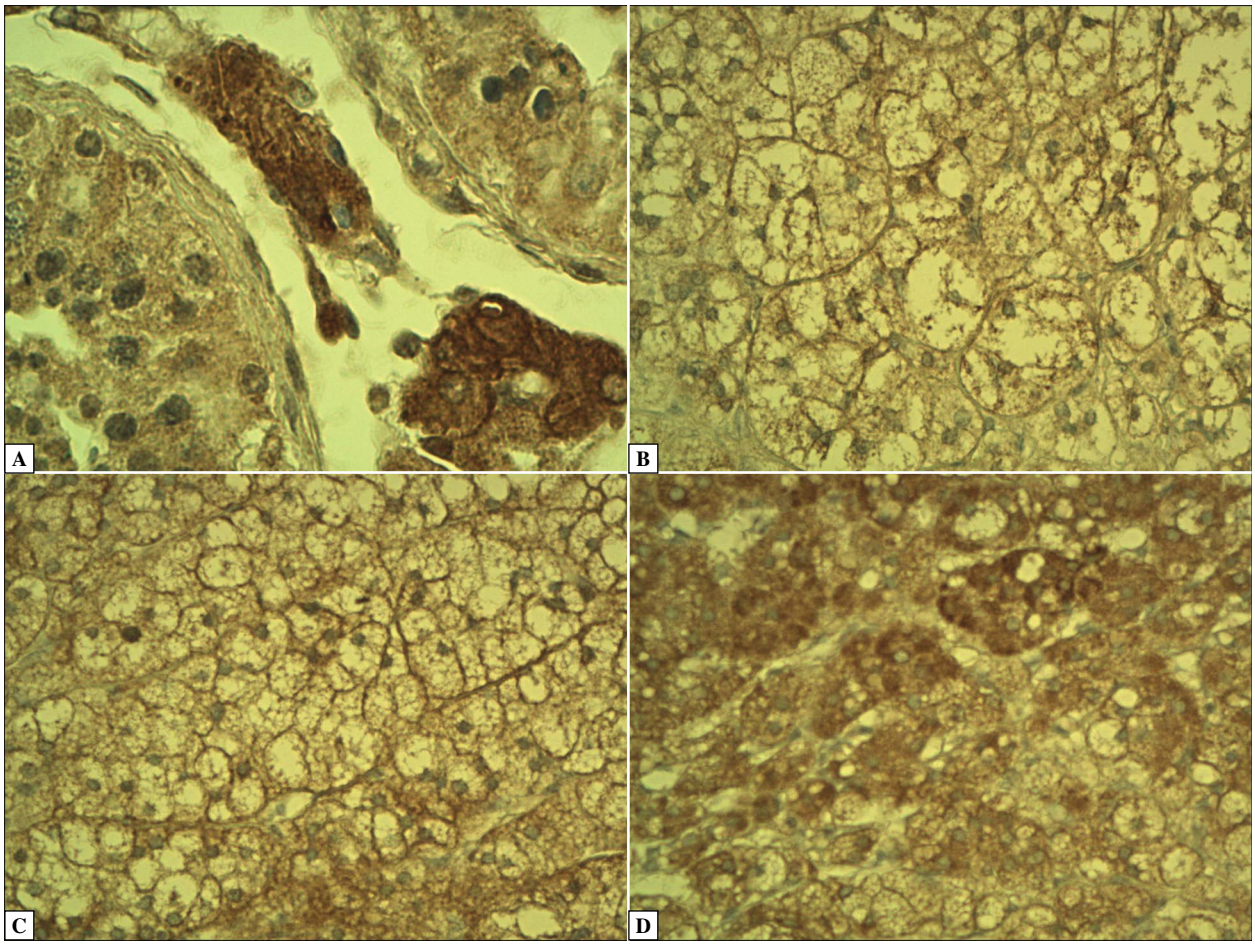


**Figure 1.** Immunostaining of LH/CGR in human non-neoplastic adrenocortical samples and testis. **A.** Positive control, human Leydig cells, 400×. **B.** Non-neoplastic adrenal cortex, glomerular zone, 200×. **C.** Non-neoplastic adrenal cortex, fascicular zone, 200×. **D.** Non-neoplastic adrenal cortex, reticular zone, 200×.

biology techniques [4–13]. Although these studies present a high level of credibility, the information on precise tissue localization of LH/CGR is insufficient. Such data could be only supplied by morphological methods, mainly by immunohistochemistry. However, the immunohistochemical studies on the localization of LH/CGR in human adrenal cortex and adrenal tumors are very scarce. Lesley *et al*. [14] investigated the presence of LH/CGR in the normal adrenal cortex of Rhesus macaque. They found, like we did in the humans, the positive immunoreactivity for LH/CGR in all adrenocortical zones. It is also worth recalling that we found similar immunopositivity in the normal adrenal cortex and adrenal tumors using the antibody for follicle stimulating hormone receptors (FSHRs) [15].

The LH/CGR immunopositivity which we found in adrenal benign and malignant tumors is in agreement with the results of previous studies [6–11]. Because the LH/CGR expression takes place also in the normal adrenal cortex, there is no reason to call them „ectopic". The sharp difference in the topography of LH/CGR immunoreaction between the benign and malignant adrenal neoplasms is worth to underline. Because of that the immunohistochemical examination of LH/CGR seems to be useful in the differentiation between benign and malignant tumors of the adrenal cortex during the histopathological diagnosis. To our best knowledge, this finding has not been reported previously in the literature. However, our findings need to be confirmed on the larger material. Moreover, since LH/CGR belongs to membrane receptors, the partial or total loss of their membrane localization in adrenocortical cancers suggests the alteration of their function. The described loss of membrane localization may reflect either down-regulation due to decreased expression of receptor gene at transcriptional level or the increased internalization of the receptor protein resulting from the ligand excess. The further studies, especially *in vitro*, concerning these possibilities are needed. The role of the direct action of LH

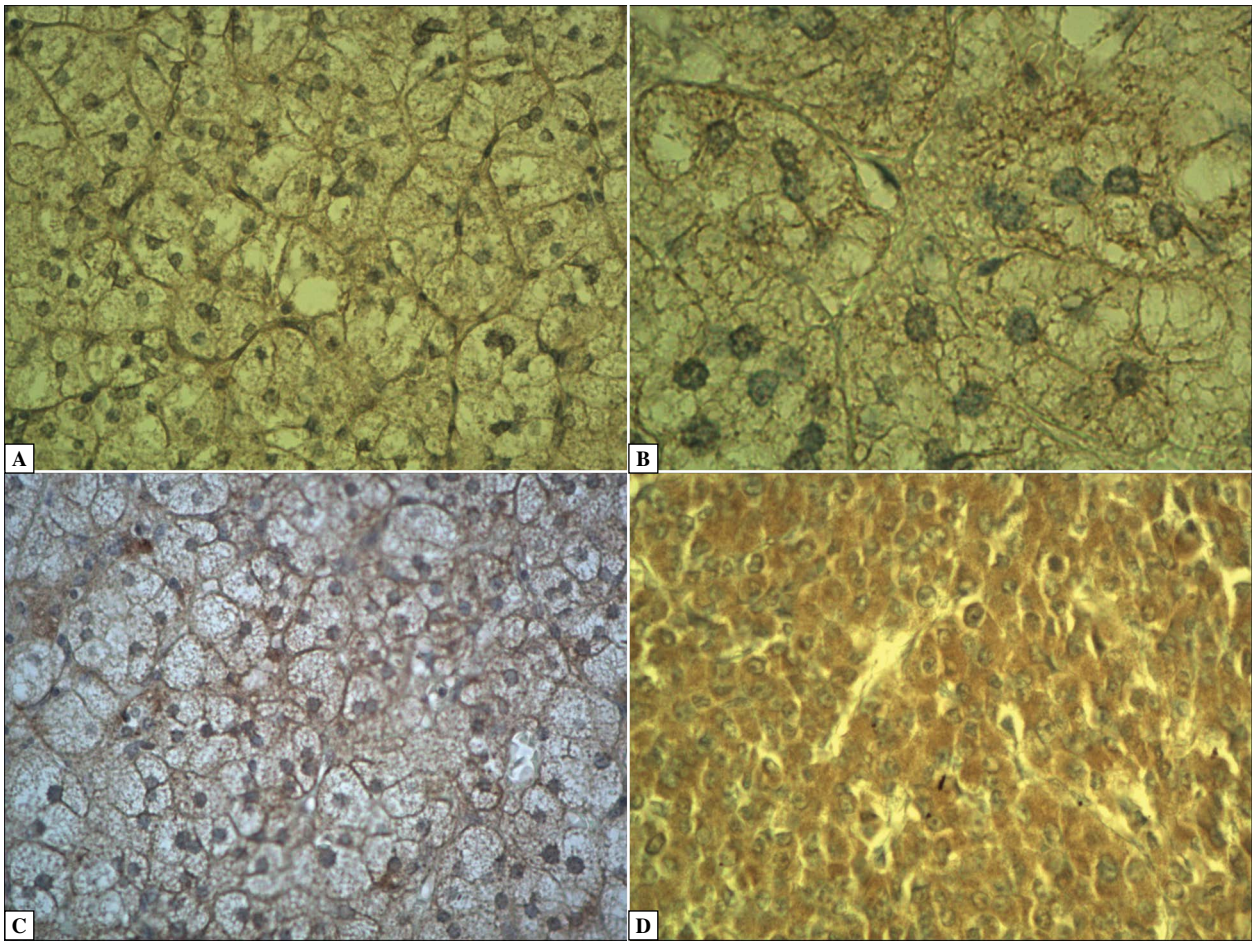


**Figure 2.** Immunostaining of LH/CGR in human neoplastic adrenocortical samples. **A.** Non-functioning adrenocortical adenoma, 200×. **B.** Cortisol-secreting adrenocortical adenoma (Cushing syndrome), 400×. **C.** Aldosterone-secreting adrenocortical adenoma (Conn's syndrome), 200×. **D.** Hormonally non-functioning adrenocortical cancer, 200×.

in adrenal cortex is not fully understood. It probably acts as an additional regulator of steroidogenesis and adrenocortical cell growth. Its involvement in adrenocortical tumorigenesis has been also suggested and proved on certain animal models [16, 17].

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ORIGINAL PAPER

VIA MEDICA

# The role of CD44H molecule in the interactions between human monocytes and pancreatic adenocarcinoma-derived microvesicles

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**Abstract**

**Introduction.** CD44H is a transmembrane molecule important for cell-cell and cell-extracellular matrix interactions. In monocytes, CD44H is implicated in phagocytosis of particles coated by hyaluronan (HA). HA fragments were shown to induce chemokine secretion by monocytes. Tumour derived microvesicles (TMVs), which are small membrane fragments derived from tumour cells can carry fragments of HA. The aim of the study was to examine whether monocyte's CD44H is involved in the engulfment of pancreatic adenocarcinoma-derived microvesicles and in the production of chemokines induced by TMVs.

**Materials and methods.** TMVs engulfment and chemokines' secretion stimulated with TMVs were determined in control human monocytes and cells incubated with anti-CD44H monoclonal antibody (mAb) by flow cytometry and ELISA, respectively. Phosphorylation of STAT3, transcription factor essential for chemokines' production and CD44 signal transduction, was determined by Western blotting.

**Results.** Blocking of CD44H by anti-CD44H mAb on monocytes decreased the engulfment of TMVs and the secretion of CCL4 and CCL5, but had no effect on CCL2, CCL3 and CXCL8. STAT-3 phosphorylation in monocytes incubated with TMVs after CD44 blocking was also reduced.

**Conclusion.** The results suggest that tumour-derived microvesicles (TMVs) may carry bioactive cargo(s) which induces STAT3 dependent signalling pathway in human monocytes *via* CD44 molecules. *(Folia Histochemica et Cytobiologica 2019, Vol. 57, No. 1, 28–34)*

**Key words:** CD44; human monocytes; HPC-4 cells; tumour-derived microvesicles; chemokines; STAT3 phosphorylation; flow cytometry

## Introduction

CD44 is a transmembrane glycoprotein expressed on different cell types, including epithelial [1, 2], hematopoietic and cancer cells [3–6]. CD44 is expressed mainly in a short, standard form (CD44s or CD44H) or in alternatively spliced variant forms (CD44v) [7, 8]. Expression of CD44H on monocytes is very high (above 90%) [9]. CD44v is almost absent on monocytes of healthy humans; however, its expression (CD44v3, -v6, -v7) is upregulated in inflammatory diseases (*e.g.* systemic *lupus erythematosus,* inflammatory bowel disease) [10, 11], malignancies (*e.g.* CD44v3, v6, v7, v10) [9, 12–14] or co-cultures of monocytes with tumour cells (*e.g.* pancreatic adenocarcinoma cell line, CD44v6, v7/8) [15].

The increased expression of CD44v molecules on cancer cells is usually associated with upregulation of tumour growth, metastasis formation and poor prognosis in cancer patients [14]. CD44 molecule is

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important in cell-cell and cell-extracellular matrix interactions, as CD44 is a receptor for hyaluronan (hyaluronic acid, HA), collagens, osteopontin, serglycin, fibronectin and laminin [16]. HA carried by tumour cells seems to be, at least in part, responsible for the stimulation of cytokines and chemokines production by monocytes [17, 18]. Apart from direct stimulation by tumour cells (*e.g. via* HA and other ligands), monocytes may be stimulated by tumour-derived microvesicles (TMVs) [19]. TMVs are small membrane fragments released by tumour cells during cell proliferation, migration, activation and apoptosis [20]. TMVs express CD44s and CD44v and carry HA [19, 21]. TMVs may mimic activity of tumour cells, as they have been shown to induce cytokines' (TNF, IL-10, IL-12), chemokines' (CXCL8, CCL2, CCL3, CCL4 and CCL5) and reactive oxygen intermediates (ROI) production by monocytes [19, 22]. It was reported that TNF production in monocytes was CD44-dependent [19] and that IL-10 production by classical monocytes was induced by low molecular weight hyaluronan carried by TMVs [21]. HA-CD44 interaction promotes phosphorylation of STAT3 [23]. CD44 is also described as fully competent phagocytic receptor able to trigger engulfment of large particles by macrophages [24].

The current study was designed to extend the knowledge on the role of CD44H in monocyte-TMVs interactions. We focused on the engulfment of TMV derived from HPC-4 cell line ($TMV_{HPC}$) and the secretion of selected chemokines (CXCL8 (IL-8), CCL-2 (MCP-1), CCL3 (MIP-1$\alpha$), CCL4 (MIP-1$\beta$) and CCL5 (RANTES)) previously described to be induced by TMVs [22]. The present study shows that blocking of monocytic CD44H molecule with anti-CD44 monoclonal antibody (mAb) reduced $TMV_{HPC}$ engulfment and decreased secretion of CCL4 and CCL5 but not CCL2, CCL3 and CXCL8. We also proved the contribution of STAT3 in signalling pathway initiated by CD44H-TMVs interaction.

## Materials and methods

**Isolation of tumour derived microvesicles (TMVs).** TMVs were obtained from the HPC-4 cell line (human pancreatic adenocarcinoma) $TMV_{HPC}$, [25] as described previously [26]. Briefly, cells were cultured by bi-weekly passages in RPMI 1640 medium (Sigma, St. Louis, MO, USA) with 5% FBS (foetal bovine serum, PAA Laboratories, Pasching, Germany). The cell line was regularly tested for *Mycoplasma sp.* contamination by using the PCR-ELISA kit according to the manufacturer's protocol (Roche, Mannheim, Germany). For the final cultures, FBS centrifuged at 50000 *g* was used (for 1 h at 4°C). Supernatants from well-grown cell cultures were collected and spun down at 2000 *g* for 20 min at room temperature (RT) to remove cellular debris. Then, supernatants were again centrifuged at 50000 *g* for 1 h at 4°C. Pellets were washed several times to remove FBS and were finally resuspended in serum-free RPMI 1640 medium. Quantification of $TMV_{HPC}$ proteins was evaluated by the Bradford method (BioRad, Hercules, CA, USA). $TMV_{HPC}$ were tested for endotoxin contamination by the Limmulus test according to the manufacturer's instruction (Charles River Laboratories, Inc., Wilmington, MA, USA) and stored at –20°C.

**Isolation and culture of monocytes.** Human peripheral blood mononuclear cells were isolated from EDTA-blood of healthy human donors by the standard Ficoll/Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were separated from mononuclear cells by counter-flow centrifugal elutriation with a JE-5.0 elutriation system equipped with a 5 ml Sanderson separation chamber (Beckman, Palo Alto, CA, USA), as previously described [27]. Monocytes were suspended in RPMI 1640 culture medium supplemented with L-glutamine (Sigma) with gentamycin (25 $\mu$g/ml). Purity of isolated monocytes was above 95% as judged by staining with anti-CD14 mAb (BD Biosciences Pharmingen, San Diego, CA, USA) and flow cytometry analysis (FACS Calibur, BD Biosciences Immunocytometry Systems, San Jose, CA, USA). Monocytes ($1 \times 10^6$/ml) were cultured with $TMV_{HPC}$ (30 $\mu$g/ml) in low attachment culture plates (Corning Inc., Corning, NY, USA) in RPMI 1640 medium supplemented with 10% FBS (centrifuged as above) as described before [19].

**Engulfment of TMVs by monocytes.** $TMV_{HPC}$ were incubated for 5 min with red PKH26 dye (Sigma) according to the manufacturer's instructions. Next, $TMV_{HPC}$ were washed with 1% bovine serum albumin (BSA) and several times with serum-free RPMI 1640 medium. Monocytes ($1 \times 10^6$/ml) were incubated with anti-CD44 mAb (10 $\mu$g/ml, clone SFF-2, BenderMedsystem, Vienna, Austria) or appropriate IgG1 isotype control (10 $\mu$g/ml Bender Medsystem) for 2 h at 37°C followed by washing and incubation with a fluorescent dye PKH26-labelled $TMV_{HPC}$ (30 $\mu$g/ml) (30 min to 24 h at 37°C in serum-free medium). Binding of PKH26-labelled $TMV_{HPC}$ to control and CD44-blocked monocytes was determined by flow cytometry analysis of red fluorescence intensity (emission at 567 nm) and calculation of the percentage of positive cells. Vital dye crystal violet was used for quenching extracellular fluorescence [28].

**Determination of chemokines' secretion by monocytes incubated with TMVs after blocking of CD44 molecule on monocytes.** To determine its role in monocyte-$TMV_{HPC}$ interactions, the blocking mAb against CD44H was used. Monocytes were incubated on 96 microwell plates with

anti-CD14 mAb (10 μg/ml, clone MY4, Coulter Corp., Miami, FL, USA) or appropriate IgG1 isotype control for 2 h at 37°C. Then, monocytes were washed and cultured with $TMV_{HPC}$ (30 μg/ml) for 18 h. Next, the supernatants were collected and chemokines' (CXCL8, CCL2, CCL3, CCL4 and CCL5) concentration was assessed by the FlexSet method (BD Biosciences Pharmingen) according to the manufacturer's protocol. The FlexSet beads were discriminated in FL-4 and FL-5 channels, while the concentration of specified chemokine was determined by the intensity of FL-2 fluorescence. The concentration of chemokines was computed by using the respective standard reference curve and FCAP Array software (BD Biosciences). For all the tested chemokines the detection level was 10 pg/ml.

**Western blotting.** Monocytes were preincubated in the medium alone or with anti-CD44 mAb (10 μg/ml) for 2 h followed by washing and incubation with $TMV_{HPC}$ (30 μg/ml) for 30 min (37°C, 5% $CO_2$), then lysed in M-Per lysing buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitors (Sigma). The extracted proteins (20 μg) were loaded on 4% loading gel, electrophoresed in 12% SDS gel, and transferred to the polyvinylidene fluoride membranes (Immune-blot PVDF, 2 μm, BioRad). Phosphorylation of STAT-3 (Signal Transducers and Activators of Transcription, Tyr 705, #9131) protein was detected with rabbit polyclonal anti-phospho-STAT-3 antibody (Cell Signaling Technology, Beverly, MA, USA) and with horseradish peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA). The equivalence of protein loading was evaluated by treating the membranes with stripping buffer (Restore Western Blot Stripping Buffer, Pierce) and incubation with total anti-STAT-3 antibody (clone #9132, Cell Signaling). Blots were developed with the SuperSignal West Pico Chemiluminescent Substrate (Pierce), dried and subsequently exposed to HyperFilm (Amersham Life Science, Little Chalfont, UK).

**Statistical analysis.** Statistical analysis was performed by nonparametric Mann-Whitney test. Differences were considered significant at $p < 0.05$.

## Results

### *The role of CD44H in TMVs engulfment by monocytes*

Engulfment of $TMV_{HPC}$ was significantly reduced when incubation with $TMV_{HPC}$ was preceded by blocking CD44 on monocytes for 2 h. We did not observe significant changes after 30 min (data not shown); however, after 2 h and 24 h, the $TMV_{HPC}$ engulfment was reduced by half (Fig. 1). Appropriate isotype control did not diminish $TMV_{HPC}$ engulfment (data not shown).

### *CD44H is important for chemokines' secretion by monocytes*

The release of CCL5 (Fig. 2A) and CCL4 (Fig. 2B) from human monocytes incubated with $TMV_{HPC}$ overnight (18 h) was decreased when CD44H, but not CD14 (data not shown), was blocked by the preincubation of cells with appropriate mAb. We did not observe significant changes in the levels of CCL2 (Fig. 2C), CCL3 (Fig. 2D) and CXCL8 (Fig. 2E) after CD44 blocking.

### *The role of CD44H molecule in signal transduction*

$TMV_{HPC}$ induced phosphorylation of STAT-3 proteins in monocytes. Phosphorylation of STAT-3, as determined by Western blotting, was reduced when monocytic CD44H was blocked with anti CD44mAb before stimulation with $TMV_{HPC}$. Results of one representative experiment out of three performed is presented in Figure 3.

## Discussion

Monocytes and TMVs derived from CD44-positive tumour cell lines express CD44H molecules [29, 30]. TMVs can also carry HA derived from cancer cells [21]. Interaction between monocytes' CD44 molecule and HA carried by TMVs results in the activation of monocytes [19, 21]. Previously, we showed that monocytes activated with TMVs secreted more TNF, IL-10 and IL-12p40 and expressed higher levels of these cytokines' mRNA compared to the control [19]. The inhibitory effect of anti-CD44H mAb provided evidence that this molecule is important for TNF secretion by monocytes stimulated with TMVs [19]. Current data add to the knowledge about the role of CD44 in interactions between monocytes and TMVs. CD44 molecule seems to be important in the process of $TMV_{HPC}$ engulfment, which is strongly inhibited by blocking monocytic CD44 with anti-CD44 mAb. This observation is in concordance with the report that demonstrated inhibition by anti-CD44 mAb of erythrocytes' phagocytosis by murine macrophages [31]. Moreover, CD44 is considered as a phagocytic receptor that effectively recognizes and ingests HA-coated particles [24]. CD44 not only mediates the phagocytic mode of internalization but it also facilitates the HA-controlled uptake of a gene vector in CD44 positive tumour cancer cell lines *via* micropinocytosis [32]. The mechanism by which CD44 is engaged in this process is unclear; however, its involvement in the first step of interaction (binding) was suggested [33]. In our current study, the engulfment of $TMV_{HPC}$ was not completely reduced by anti-CD44 mAb, which may suggest other mechanisms and sur-

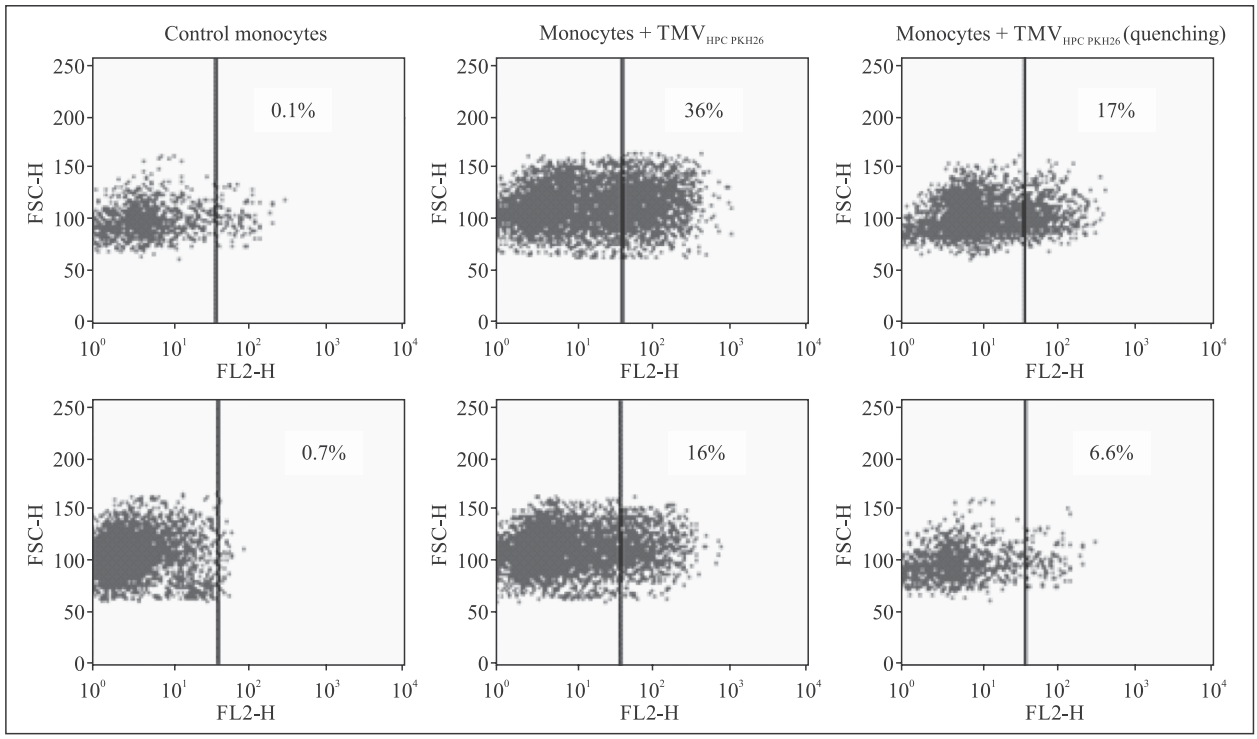


**Figure 1.** Transfer of PKH26 labelled $TMV_{HPC}$ to monocytes in the absence (upper dot plot panel) and presence (lower dot plot panel) of anti CD44H mAb (10 μg/ml). Monocytes were exposed to $TMV_{HPC}$ for 24 h and incubated either in the medium alone or with crystal violet (right panel). One representative experiment of four independent experiments is presented. Flow cytometry was performed as described in Methods.

face molecules to be involved in their internalization, *e.g.* phosphatidylserine [34]. To our best knowledge, the presented data, for the first time, provide the evidence of the role of CD44 in the engulfment of TMVs in a manner similar to that suggested for particles coated with HA [32].

McKee *et al.* [17] showed that low molecular weight HA induced the expression of mRNA for CCL2, CCL3, CCL4, CCL5 and CXCL8 in alveolar macrophages [17] and that small fragments of HA were able to induce secretion of CCL2, CCL3, CCL4 and CCL5 by these cells. These authors also demonstrated that anti-CD44 mAb completely blocked HA binding and significantly inhibited HA-induced expression of CCL4 and CCL5 in this cell type [17]. In parallel, we reported that TMVs of different origin (lung, pancreas and colon cancer cell lines) induced expression of chemokines mRNA followed by secretion of chemokines by human monocytes [22]. Keeping in mind that $TMV_{HPC}$ carry low molecular weight HA [21], we blocked CD44H on human monocytes and observed the reduction of CCL4 and CCL5 secretion by monocytes. The incomplete reduction in chemokines secretion that we have seen may be a result of the contribution of other HA receptors, which were not blocked by the anti-CD44H mAb used. Levesque *et al.* described that blood monocytes up-regulated CD44v6 and v9 expression after *in vitro* culture [35]. Also, co-culture with tumour cells induced expression of CD44v6 and v7/8 on human monocytes [15]. Thus, in our study, the mAb specific for CD44H blocking may have not blocked CD44v, which in turn could have resulted in HA binding. Also, other molecules, such as TLR4, have to be taken under consideration as HA receptors [36]. Moreover, lipids, heat shock proteins [37] or nucleotides [38] carried by TMVs may be involved in the induction of chemokines. Other components of TMVs may address the question about the role of CD44H in the signalling pathway for the chemokines which secretion was unaffected by anti-CD44 mAb and TMVs (CXCL8, CCL2 and CCL3). Another possible explanation is horizontal transfer of chemokines by TMVs, as TMVs are a storage pool for CC and CXC chemokine families [22, 39].

It was reported that phosphorylation of STAT3 transcription factor is important for the synthesis of chemokines by monocytic cells [40–42] or vascular smooth muscle cells [43]. $TMV_{HPC}$-induced STAT-3 phosphorylation in monocytes was shown to be involved in TNF and IL-10 gene transcription [19].

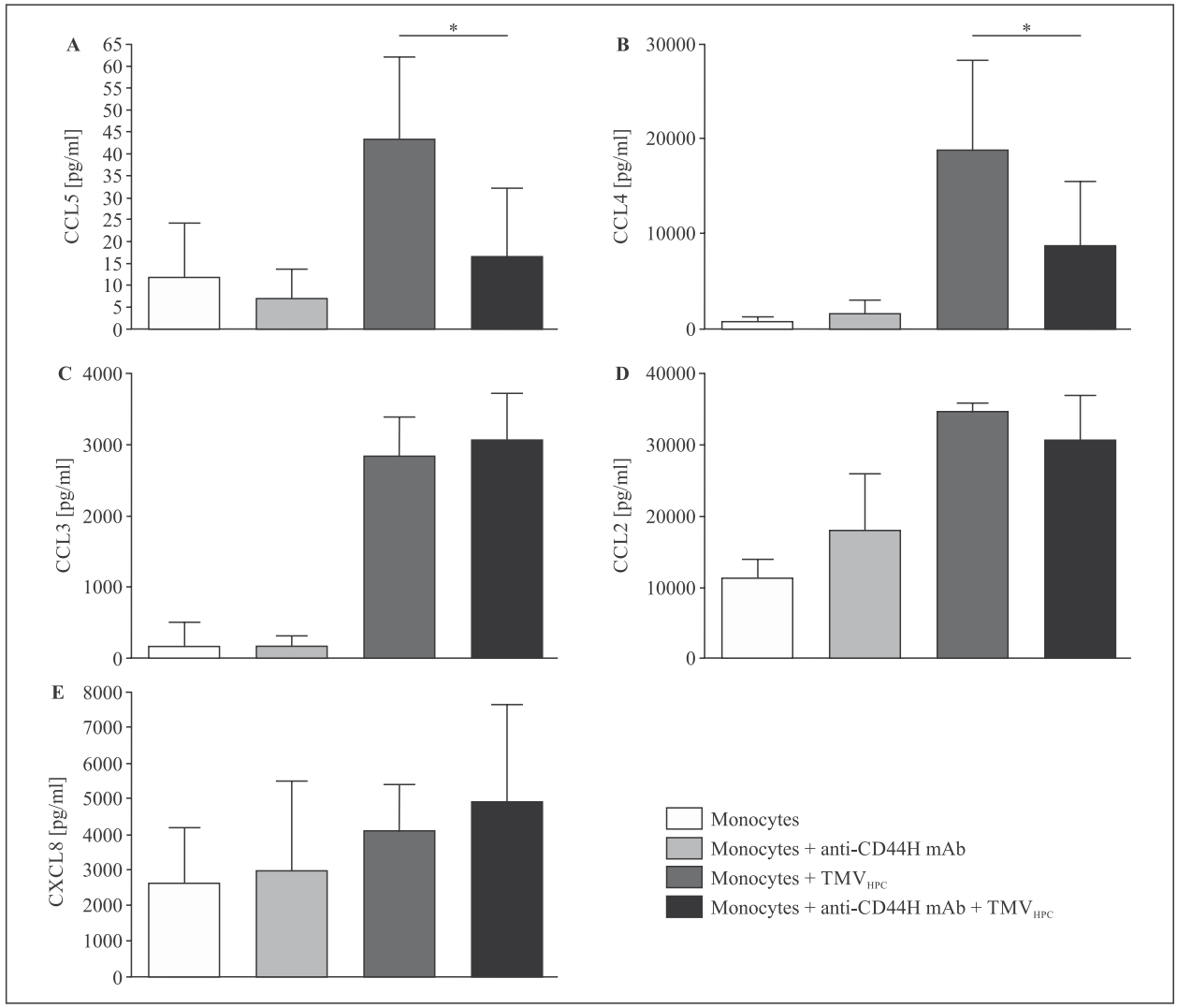


**Figure 2.** Secretion of chemokines by monocytes stimulated with $TMV_{HPC}$ alone or in the presence of anti-CD44H mAb (10 $\mu$g/ml). A-CCL5, B-CCL4, C-CCL3, D-CCL2, E-CXCL8. Concentration of chemokines was measured by the FlexSet method as described in Methods. Data presented as mean ± SD of five independent experiments. * $p < 0.05$.

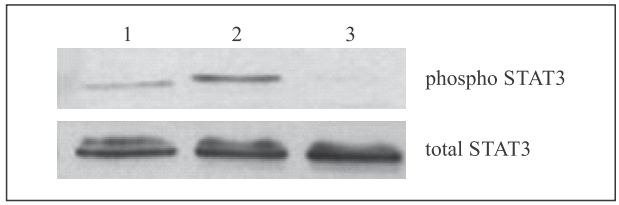


**Figure 3.** Expression of phosphorylated (Tyr 705) and total STAT-3 determined by Western blotting in unstimulated monocytes (lane 1) or in monocytes stimulated for 30 min with $TMV_{HPC}$ (lane 2) alone or after preincubation with anti-CD44H mAb (lane 3).

Reduction in STAT3 phosphorylation after CD44H blocking may suggest that this signalling pathway is induced in monocytes by $TMV_{HPC}$. Taken together, the results of this study point out to the role of CD44 in $TMV_{HPC}$ –monocyte interaction. $TMV_{HPC}$ carry information which is, at least partially, passed through the signalling pathway initiated by CD44 molecules. $TMV_{HPC}$, may be considered as an important chemokine secretion trigger during tumour progression. The presented data imply that TMVs may play a role in the communication between various types of cells, including tumour cells, at local and distant levels.

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## Competing interests

The authors have no conflicts of interest.

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CASE REPORT

VIA MEDICA

# The role of immunohistochemical examination in diagnosis of papillary thyroid cancer in struma ovarii

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**Abstract**

**Introduction.** Struma ovarii (SO) is a monodermal teratoma in which thyroid tissue comprises more than 50% of the tumour. Papillary thyroid cancer (PTC) in SO is a rare finding, as only 5% of SO cases undergo malignant transformation. Malignant SO is usually asymptomatic and infrequently diagnosed preoperatively. Because of its rarity, there is no consensus about diagnosis and management, while treatment and follow-up procedures are not clearly established.

**Material and methods.** Herewith, we report two cases of PTC in SO. The first patient was a 25-year-old woman diagnosed with bilateral ovarian tumours. The second patient, 19-year-old woman, presented with unilateral ovarian mass. Both patients were qualified for surgical excision of the tumours. Histopathological specimens underwent both conventional histopathological assessment and immunohistochemical staining.

**Results.** In the first patient histopathology revealed SO with two foci of PTC. Immunohistochemically a positive expression of CK7, CK19, p63 and thyroglobulin (Tg) confirmed the diagnosis. She underwent total thyroidectomy in 2016 in order to enable ablative radioiodine therapy and facilitate further thyroglobulin monitoring. Unfortunately, the patient was lost from follow-up. In the second patient, histopathological diagnosis was follicular variant of PTC in SO. Postoperatively, a pelvic CT revealed osteolytic lesion 6 cm in size, being a metastatic change. The patient underwent unilateral ovariectomy, total thyroidectomy and multiple cycles of radioiodine therapy. Currently, 9 years following the diagnosis, the patient achieved disease remission.

**Conclusions.** PTC in SO still remains a diagnostic and therapeutic challenge. Immunostaining for CK7, CK19, p63 and Tg might be helpful in histopathological diagnosis. The decision on the need of total thyroidectomy and radioiodine therapy should be made individually. However, thyroid remnant ablation increases the sensitivity and specificity of follow-up testing using serum Tg level as a tumour marker. *(Folia Histochemica et Cytobiologica 2019, Vol. 57, No. 1, 35–41)*

**Key words:** struma ovarii; papillary thyroid cancer; teratoma; CK7; CK19; p63; thyroglobulin; IHC

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## Introduction

Germ cell tumours are a heterogeneous group of neoplasms arising from primordial germ cells. The most common type of germ cell tumours are teratomas [1]. Struma ovarii (SO) is a monodermal germ cell tumour characterized by the presence of thyroid

tissue in more than 50% [2]. It was first described in 1899 by Boettlin, who observed the presence of thyroid follicular tissue in the ovaries [3]. It is most often diagnosed postoperatively during histopathological examination, while radiological imaging is unspecific [4]. Strumae ovarii are one of the rarest ovarian tumours — they represent 1% of them and comprise 2–5% of teratomas [5]. Less than 200 cases have been so far reported in medical literature [2]. Struma ovarii is usually a benign tumour, being histologically and pathologically identical to normal thyroid tissue found in typical localization. Less than 5% of these tumours undergo malignant transformation [2, 6–8]. The age at presentation of SO is usually between 40 and 60 years [3, 9, 10]. The most common type of cancer within SO is well-differentiated thyroid cancer, most commonly, papillary thyroid cancer (PTC) [9, 11]. The diagnosis of thyroid cancer arising in SO should be based on the similar criteria as for primary thyroid gland disease.

Due to the rarity of this type of tumour, there has been a paucity of data in the medical literature about its optimal diagnosis, treatment and follow-up. Classically, the treatment is the surgical resection of the ovarian mass. Iodine-131 therapy can be proposed after total thyroidectomy, but no consensus exists in terms of surveillance and adjuvant treatment [12]. Diagnostic difficulties may appear also at the level of histopathological examination. In this report we present two patients diagnosed postsurgically with thyroid cancer in SO and demonstrate the pivotal role of immunohistochemical (IHC) staining for final diagnosis.

## Material and methods

Herewith we report two cases of PTC in SO. The first patient was a 25-year-old woman diagnosed with bilateral ovarian tumours. She was suffering from recurrent acute lower abdominal pain. Similar episodes of pain were experienced during sexual intercourses in the previous two years, since she gave birth to a first child via caesarean section. There were no other associated gastrointestinal or genitourinary symptoms. She was otherwise healthy with nonsignificant past medical history. The second patient, 19-year-old woman, presented with unilateral ovarian mass. Both patients underwent clinical assessment, imaging studies and were qualified for surgical excision of the ovarian tumours. Histopathological specimens underwent both conventional histopathological assessment and IHC staining.

**Immunohistochemistry.** Formalin fixed, paraffin embedded tissue specimens were cut into 4 $\mu$m sections. The sections were mounted on adhesion microscope slides (Dako, Glostrup, Denmark). Deparaffinization, rehydration and antigen retrieval was achieved by incubation in a water bath (PT Link, Dako) in a buffer (High pH, EnVision FLEX Target Retrieval Solution, Dako) at 97°C for 20 min. The sections were then machine stained with the use of Autostaner Link 48 using a visualization kit for IHC staining (EnVision FLEX+, Mouse, Dako). Prediluted primary antibodies included: mouse monoclonal anti-Human Cytokeratin 7 (clone: OV-TL 12/30), monoclonal mouse anti-human cytokeratin 19 (clone RCK108, Dako), monoclonal mouse anti-human p63 protein (clone: DAK-p63), polyclonal rabbit anti-human thyroglobulin (Dako), and Rabbit Linker (Dako) for amplification. Then, the slides were dehydrated and mounted with cover glass with Mounting Medium (Dako).

## Results

In the first patient, an ultrasound examination revealed normal sized and shaped uterus and endometrial thickness of 4 mm. Moreover, left ovarian cyst 4.7 cm in size with dense content and 8.7 cm mass behind the uterus suggestive of a teratoma of a right ovary, were revealed. Ultrasound pattern was compatible with presumably benign tumours; hence, there was no need to conduct computed tomography (CT) scans or magnetic resonance imaging (MRI). The patient was qualified for surgery; due to the size and bilateral location of the lesions, the patient was qualified for conventional laparotomy. During surgical procedure, abdominal cavity and uterus were normal, while the ovaries were enlarged and presented bilateral masses which were enucleated and removed. Intraoperative histopathological examination revealed a teratoma on the right side (9.0 × 6.0 × 5.0 cm) with SO and dermoid cyst in the left ovary (8.0 × 7.8 × 2.0 cm). In the right ovary mass the biggest cyst was 3 cm in size and was filled with clear serous fluid. In the other cysts there was yellow, viscous material. Patient's postoperative period was uneventful.

Microscopic examination of the surgical specimen prepared by haematoxylin and eosin (H&E) staining (Figs. 1A, B) revealed two foci of PTC in SO (the largest 4 mm in size). The diagnosis was confirmed by an IHC examination. Immunohistochemically, tumour cells were strongly positive for thyroglobulin (Tg), cytokeratin 19 (CK19), cytokeratin 7 (CK7) and p63 (Figs. 2A–D). Based on all these findings, the diagnosis was papillary thyroid cancer arising within SO. Subsequently, in order to enable potential ablative radioiodine therapy and facilitate further Tg monitoring, the patient was referred for total thyroidectomy. The patient had two mixed solid/cystic focal lesions in the thyroid (size 8 mm and 11 mm) detected on thyroid ultrasound examination. Both presented

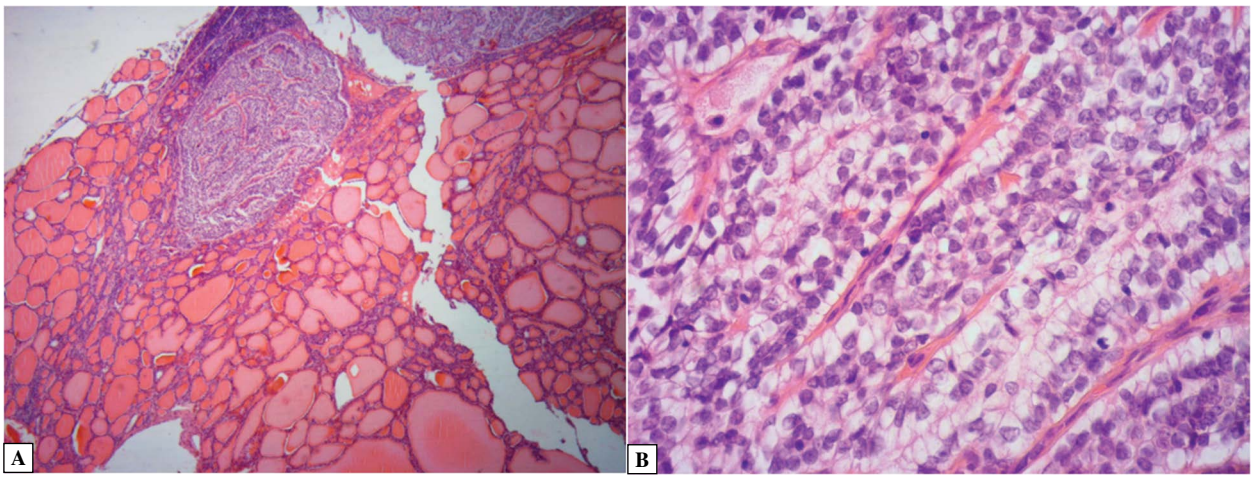


**Figure 1.** Microscopic view of papillary thyroid cancer in struma ovarii of the Patient 1. H&E staining. Total magnification **A.** 40× and **B.** 400×.

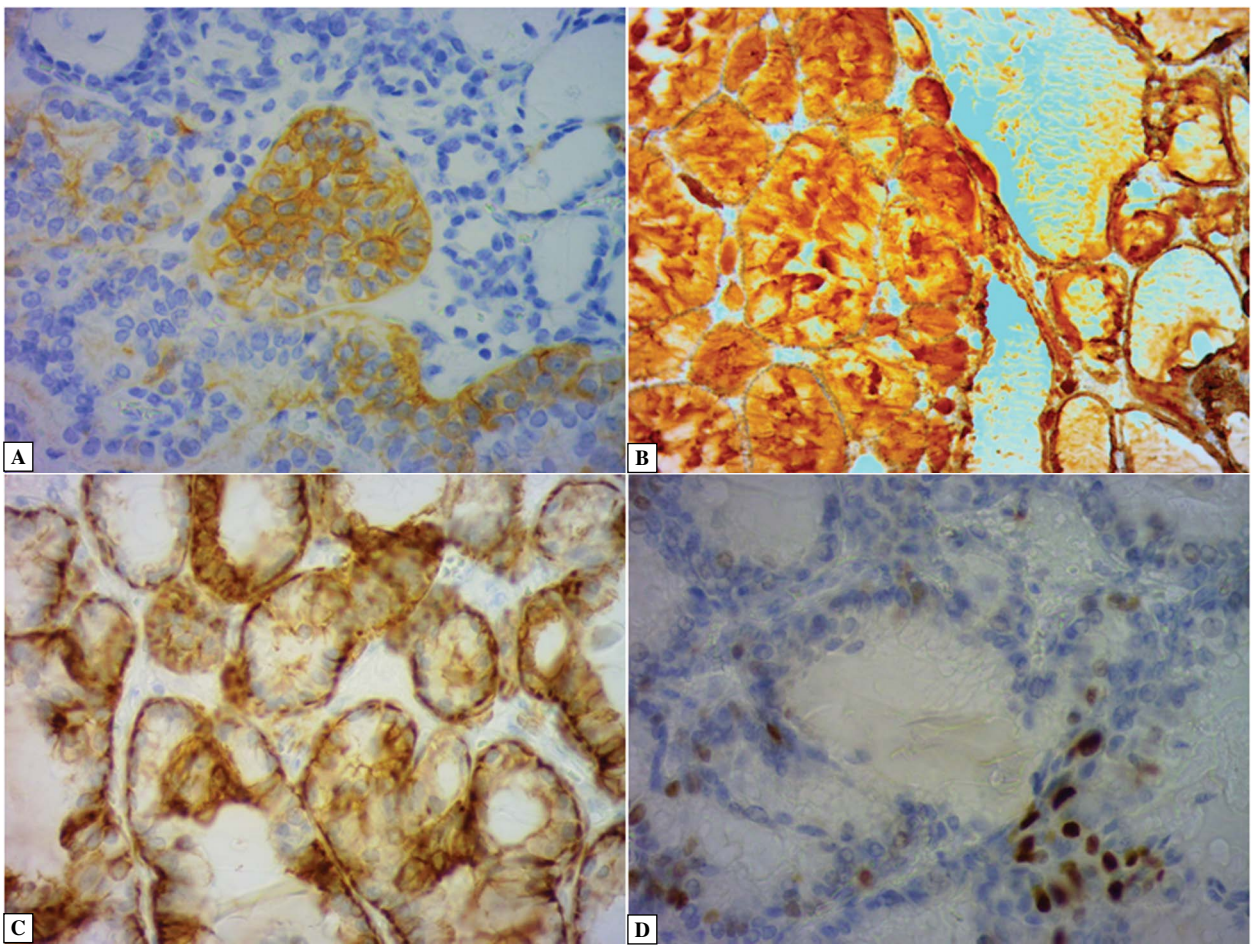


**Figure 2A.** Immunohistochemical (IHC) detection of the studied antigens in the tissue of papillary thyroid cancer in struma ovarii of the Patient 1. **A.** CK19 positive immunoreactivity in the tumor tissue, while negative in the surrounding non-cancerous thyroid tissue. **B.** Immunoreactivity of thyroglobulin. **C.** Immunoreactivity of cytokeratin 7. **D.** Immunoreactivity of p63. Immunohistoch-emical stainings were performed as described in Methods. Total magnification 400×.

no sonographic features of malignancy and were diagnosed as benign colloid nodules on cytological examination of the specimen obtained on fine-needle aspiration biopsy. On postsurgical histopathological examination there were no malignant lesions found in the thyroid, and colloid goitre was diagnosed. After surgery the patient was treated with oral L-thyroxine therapy and the post-surgical serum Tg concentration on suppressive doses measured 6 week following surgery was 0.9 ng/ml, marking the low risk of persistent disease. The patient was scheduled for whole-body scintiscan and stimulated Tg assessment 3–6 months following surgery in order to decide whether to administer radioiodine therapy. Unfortunately, the patient was lost from follow-up. Still, in this patient a long-term regular follow up is required in order to monitor Tg concentration and including regular gynaecological visits.

The second patient had an unilateral ovarian mixed solid and cystic mass (4.0 × 3.0 × 6.8 cm) diagnosed during routine gynaecological follow-up. An excision of the lesion was performed from laparoscopic approach. Histopathological diagnosis was teratoma of the size 6 × 7 × 4 cm that contained focus of follicular variant of PTC in SO (size not reported, histopathological material unavailable). Two months after the surgery, laparoscopic complete right ovariectomy was performed and two months later total thyroidectomy was performed. No focal lesions were detected in the thyroid preoperatively on ultrasound examination or postoperatively on histopathological examination. Radioiodine therapy was then introduced — 200 mCi 131-I was administered. Few months later pelvic CT scan revealed an osteolytic lesion in the left ilium measuring 4.8 × 5.8 × 1.0 cm, being a metastatic change. Whole-body scintiscan depicted radioiodine uptake in the metastasis. During 9 years of follow-up the patient was on constant L-thyroxine suppressive therapy and received cumulative activity at the level of 1400 mCi of radioiodine. At the moment she achieved complete biochemical and structural disease remission.

## Discussion and conclusions

Most patients with SO present no clinical symptoms and are diagnosed incidentally. In some patients, as it was in the two presented cases, they may cause symptoms similar to those typical of other ovarian tumours, such as low abdominal pain, nausea, vaginal bleeding and abnormal menstrual cycles [5, 6]. Sometimes, the first symptom is palpable abdominal mass [5, 7, 13]. The late manifestation of malignant SO are signs of metastatic disease such as back pain [14, 15]. Dissimilar from previous reports, in two of our patients SO was diagnosed in their 19th and 25th years of age (in comparison to average 40–60 years reported in the literature). The most common type of cancer detected in this type of teratoma is PTC, as it was in our patients. In one of them follicular variant of PTC was diagnosed at the stage of distant bone metastasis. Distant metastases from malignant SO are highly uncommon and occur in only about 5% of cases [16]. Tumours can metastasize *via* direct spread to the omentum, peritoneal cavity and contralateral ovary, *via* the lymphatic system to pelvic and para-aortic lymph nodes and *via* blood to the bones, brain, lung, contralateral ovary and liver [16]. Bone is a very rare site for metastasis and there are only a few cases in the English language literature which have reported metastasis to the bones [17–19]. The metastatic pattern of malignant SO resembles that of ovarian cancer; therefore, for advanced disease certain authors propose performing complete staging surgery as for ovarian cancer.

Similarly to both our patients, over 92% of the patients with thyroid cancer in SO are clinically and biochemically euthyroid [3, 20, 21]. However, 83% of patients who present with hyperthyroidism already had distant metastases from malignant SO [22]. There is no typical ultrasound feature of SO, although it could be suspected if there is a large solid mass on ultrasound examination, as it was in both our patients. Tumour markers are not useful and they represent low clinical value in these cases. The symptoms are also non-specific; therefore, most cases of malignant SO have subclinical course and are diagnosed incidentally. Most frequently the patients present with pelvic pain. Postsurgical assessment of thyroglobulin can be used for follow-up as a marker of response to treatment, especially in cases of metastases.

Both papillary and follicular thyroid cancers arising in SO have been reported in medical literature [16]. The most common type of malignant SO is PTC [15, 23, 24]. The pathological diagnosis of malignant SO is primarily established according to the criteria for tumours of the thyroid gland. These criteria include ground glass overlapping nuclei and nuclear grooves, or mitotic activity and vascular invasion [8, 15]. The diagnosis of thyroid cancer in SO might be challenging. The "gold standard" in diagnosis of thyroid cancers is using routine H&E staining. Immunohistochemistry is an additional procedure, often necessary to provide diagnosis. Besides H&E staining for evaluating thyroid tumour morphology, immunohistochemical methods have been widely used [25]. Immunohistochemical markers are of two types: those related to proteins present in normal

thyrocytes (such as thyroglobulin and thyroid transcription factor-1) and those related to proteins in pathological thyrocytes (such as CK 19 and Hector Battifora mesothelial epitope-1) [26, 27].

Cytokeratins (CK), mainly expressed in epithelial cells and skin appendages, constitute the largest subgroup of intermediate filament proteins [28]. They participate in the formation of the cell skeleton and play an important role in the responses to stress, mitosis, the postmitotic period, cell signalling and movement, as well as in differentiation and apoptosis [26, 29]. CK19 is mainly expressed in ductal epithelium (bile and pancreatic ducts, renal collecting ducts) and gastrointestinal epithelium [30]. Expression of CK19 might also be positive in pancreatic neuroendocrine tumours, hepatocellular cancer, cervical squamous cell cancer, thymic and lung cancers [31–34]. Malignant thyroid lesions present higher CK19 expression when compared to nonmalignant thyroid lesions [27]. In our patient, we confirmed that CK19 expression in the patient's tumour was positive suggestive of malignant process. Another marker demonstrating positive staining in our specimen was CK7. In the study by Bejarano *et al*., 79% of benign and malignant thyroid tumours were found to demonstrate positive reaction for CK7, especially all eight analysed PTCs [35].

There are only a few studies on the involvement of p63 protein in processes associated with thyroid tumour formation and they represent contradictory results. This protein is a member of p53 family of transcription factors having a major role in regulating proliferation and differentiation processes of epithelial cells [36]. Immunohistochemical expression of p63 is evident in some types of cancers including squamous cell cancer, choriocarcinoma and thyroid cancer [37–39], and was also demonstrated in our patient with PTC.

The optimal treatment guidelines for PTC arising in malignant SO are still disputable. Most authors advocate for aggressive treatment based on total abdominal hysterectomy with bilateral salpingo-oophorectomy, omentectomy and lymph node dissection [2, 6, 8, 24]. Because of permanent infertility associated with this procedure, unilateral salpingo-oophorectomy/unilateral oophorectomy in order to preserve the patients' fertility is acceptable [2, 40]. As one of our patients was nulliparous and the second patient had one child, in both gynaecologic intervention was limited to an ovarian mass excision to preserve fertility and ovarian hormonal function. It is necessary to check the contralateral ovary in order to exclude any pathological changes. Moreover, the decision on completion thyroidectomy raises controversies. Debate remains about the role of total thyroidectomy and subsequent radioiodine ablation. Most authors suggest aggressive treatment with radioactive iodine therapy regardless of the presence or absence of metastases at time of diagnosis [2, 41, 42]. Some authors agree that thyroidectomy and radioactive iodine therapy should be undertaken only in case of recurrent disease or metastases [43]. In general, completion thyroidectomy and radioiodine ablation therapy appear to be well-accepted and effective treatments for malignant SO most often reported in the literature [16, 44], which also adheres to the standards applied to manage our patients.

Thyroid-type cancers arising in SO, especially the newly recognized entity, *i.e.* highly differentiated follicular cancer of ovarian origin, have a favourable prognosis with 5- and 25-year survival rate of 92% and 79%, respectively [8, 43, 45]. Long-term follow-up with thyroglobulin and anti-thyroglobulin antibodies levels assessment is necessary due to the possibility of recurrence. Based on the rarity of these tumours and lack of firm prognostic factors, therapeutic decisions should be made individually, based on pathologic and clinical parameters, respecting the patient's informed consent.

In conclusion, PTC in SO still remains a diagnostic and therapeutic challenge. The diagnosis is most often made postoperatively. Immunostaining for CK7, CK19, p63 and thyroglobulin might be helpful in histopathological assessment. There is still no established treatment algorithm for SO patients. The decision on the need of total thyroidectomy and radioiodine therapy should be made individually. However, thyroid remnant ablation increases the sensitivity and specificity of follow-up testing using serum thyroglobulin levels as a tumour marker.

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