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Foreword

If the high rate of proliferation of new scientific journals can largely be blamed on the progressive splintering of older disciplines into ever smaller narrower technical specialties, each establishing its own house organ, the "Italian Journal of Anatomy and Embryology" has the opposite mission to promote the confluence and integration of related, but formerly isolated, lines.

The "Italian Journal of Anatomy and Embryology" will be open to papers on morphological research of any kind whatever. In the "Italian Journal of Anatomy and Embryology" will appear the following: 1. Original works from all sections of anatomy, histology, cytology, and embryology, and related subjects, the results of which have not already published elsewhere. 2. Reviews of selected subjects. 3. Reports of meetings of anatomical societies in different countries. 4. News of personal and topical interest.

Editorial policy will encourage diversity of subject matter, treatment and expression. Each contribution should be regarded as a connecting link to be utilized by other workers, so that the whole framework of facts and concepts may become ever more firmly cross-linked and stabilized.

Domenico Ribatti
Editor in Chief

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Invited reviews – Plenary lecture at the LXXII National Congress of Italian Society of Anatomy and Histology, Parma, September 2018

Anatomy of the nutritional system

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Abstract

White and brown adipocytes are contained in an anatomically dissectible structure called adipose organ. White adipocytes form white adipose tissue (WAT) brown adipocytes form brown adipose tissue (BAT). They have in common the fact that they manage lipids, but WAT uses them as an energy reserve to be given to the body between meals, while BAT burns them to produce heat. In some areas of the organ WAT and BAT are very distinct and easily recognizable by color, but in others the tissue is mixed. Numerous experimental data suggest that the concomitant presence of WAT and BAT in the adipose organ is due to the fact that they cooperate with each other thanks to their physiological and reversible transdifferentiation property. In the case of chronic cold exposure WAT converts to BAT to expand its thermogenic potential, while in the case of a chronic positive balance, BAT convert to WAT to expand the potential for energy storage. This new plastic property of physiological and reversible genetic remodeling is also present in the breast. In fact, our experimental data suggest that during pregnancy adipocytes transform into glandular epithelium that produces milk, while in the post-pregnancy period the glandular epithelium is transformed back into fat cells. The adipose organ collaborates with the digestive organs producing hormones that influence the most important of the instinctual behavioral activities: research and food intake, they also collaborate in the absorption and distribution of nutrients (both to the organism and to the offspring) and influence each other mutually for thermogenic activities that influence satiety. It can therefore be concluded that adipose organ and digestive system collaborate in a homeostatic system definable as a nutritional system.

Keywords

Adipose tissues, transdifferentiation, mammary gland, pink adipocytes, nutritional system.

Adipocyte anatomy

The term adipocyte is commonly used to define a cell rich in cytoplasmic lipids under physiological conditions.

Traditionally two types of adipocytes are described in histology books: white and brown [1, 2]. The color is assigned based on the macroscopic aspect of the tissue that contains them. The anatomy of these cells reveals a substantial difference between white and brown. In fact, white is a spherical cell whose content is mainly formed by a single droplet of triglycerides. This droplet takes up about 90% of the cell volume. The remaining 10% forms a thin cytoplasmic rim that surrounds the lipid drop and contains the squeezed nucleus. Elongated mitochondria thin with short and vari-

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ously oriented cristae without a specific morphology are observed in the cytoplasm. The other organelles are poorly represented. Numerous pinocytosis vesicles (or caveolae) are present on the cytoplasmic membrane. Externally, the latter is characterized by a typical basal membrane (or external lamina) mainly formed mainly by collagen IV. On the outer surface of the basement membrane are variously intertwined collagen fibrils that form a thin network, visible under a high-resolution scanning electron microscope (HRSEM)[3]. The size of the white adipose cell is very variable especially in relation to the location, the state of nutrition and the age of the subject [4].

The smaller mature adipocytes have a diameter of about 20-30 μm the larger ones can reach 80-100 μm in the mouse and about 30% more in humans [5].

Brown adipocytes are much smaller than white adipocytes (about 1/3-1/4) and have a polygonal shape with a rounded and often central core. In the cytoplasm many lipid vacuoles and many large mitochondria, mainly spherical, are observed. The size of the vacuoles and mitochondria is closely related to the functional state of the cell: in very active cells the vacuoles are small and the mitochondria are numerous and large and vice versa for cells that are not highly active. The cytoplasmic membrane is rich in caveolae and on the external side there is a distinct basement membrane [6].

Anatomy of adipose tissues

White fat cells are organized to form white adipose tissue (WAT) (Fig. 1). WAT is well vascularized and provided with innervation. WAT nerves contain adrenergic and sensory fibers [7, 8].

Brown adipocytes are organized to form brown adipose tissue (BAT) (Fig. 1). BAT is about six times more vascularized than WAT and has a rich innervation [9]. The fibers most represented are noradrenergic, but there are also sensitive fibers. The noradrenergic fibers infiltrate BAT and come into direct contact with the brown fat cells [10].

Physiology of adipocytes

The main function of white adipocyte is to secrete fatty acids in the intervals between meals [1, 5, 11]. This activity allows the normal functioning of the heart and has been fundamental for the survival of the human species when very long times (up to 4-6 weeks) were needed to get the next meal. The spherical shape guarantees maximum volume in the minimum space and the intrinsic energy of fatty acids is the maximum obtainable from the oxidation of the single molecules. This cell also has endocrine properties as it produces leptin, a hormone capable of influencing behavior by acting on the brain and in particular on the limbic system [12, 13]. Circulating leptin correlates positively with the amount of white fat cells in the body and therefore represents an important signal of the body's energy supply to our brain [14]. When leptinemia is low the brain is activated for a fundamental function for survival: the search for food. This is not enough to guarantee the body because the action of another hormone recently discovered and produced by white adipocytes is required:

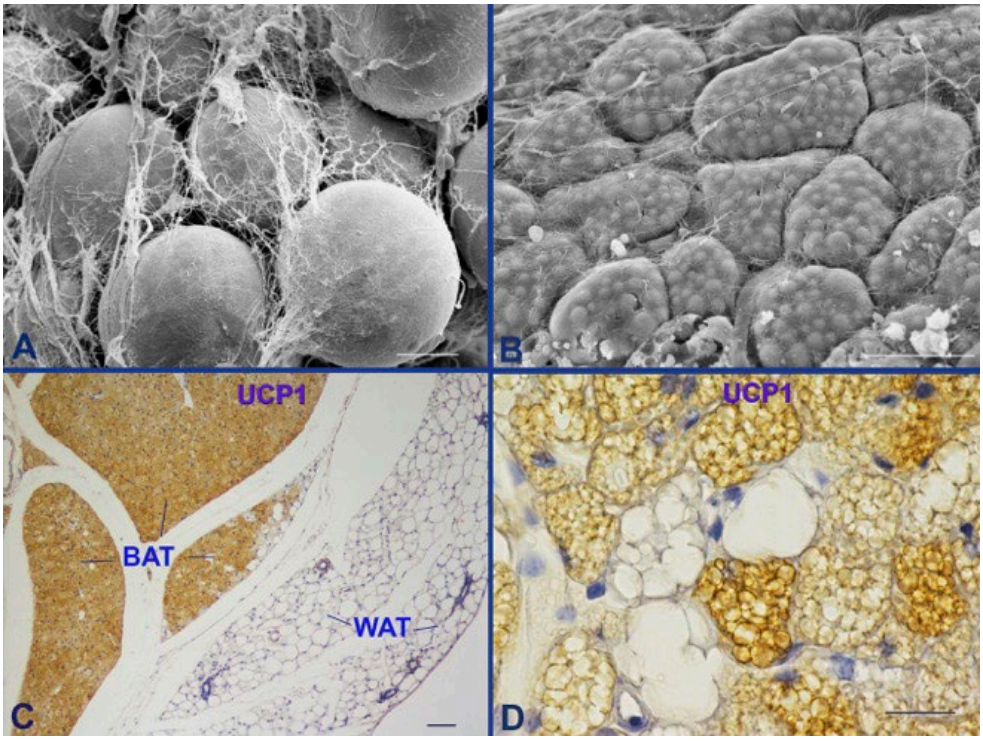

Fig 1. Morphology of murine white and brown adipose tissues. A: scanning electron microscopy of subcutaneous white adipose tissue. B: scanning electron microscopy of interscapular brown adipose tissue. C: immunohistochemistry with UCP1 antibodies showing positive brown (BAT) and negative white (WAT) adipose tissues. D: High magnification of UCP1-immunoreactive brown adipose tissue. Bar: 20 mm in A and B; 50 mm in C and D. Reproduced from [2] with permission.

asprosin [15]. The action of this last hormone on the brain is in fact necessary, and in particular on the arcuate nucleus of the hypothalamus to induce the individual to take food [16]. Subjects with mutations in the gene used to produce asprosin are lipodystrophic and therefore have low levels of leptin, but they are not hyperphagic like the majority of subjects with lipodystrophy, on the contrary they are inappetent and ingest very little food. Asprosin, in addition to directly stimulating the brain to take food, stimulates the production of glucose by the liver in order to make the fuel necessary for brain function available to the body. The main stimulus for the production of asprosin is fasting [15].

Subjects lacking functional leptin take large amounts of food from birth and the administration of recombinant leptin in these subjects returns the subject to complete normality. In addition to brain activity, leptin acts in many other organs including the gonads and the endocrine system [13, 17]. Finalistically, it can be hypothesized that the action on these organs prevents procreation to those who do not have energy supplies to guarantee the survival of newborns.

In addition to these two hormones, the white fat cell produces a series of other molecules that intervene in a series of homeostatic functions that mainly concern the glucose metabolism [18, 19]. In particular, adipsin stimulates the production of insulin and adiponectin promotes its peripheral action. A number of other endocrine, paracrine and autocrine factors are also produced by white adipocytes and recently reviewed elsewhere [1].

The main function of brown adipocyte is to produce heat. The stimulus able to induce thermogenesis is the exposure to temperatures below the thermoneutrality. The latter is variable among species: for the naked human and for the rat about 28°C, for the mouse about 34°C [9, 20-22]. Exposure to the "cold" activates the adrenergic system that causes the parenchymal fibers of the BAT to secrete noradrenaline in the neuro-adipose synaptic buttons. Norepinephrine activates specific ß3 receptors that activate protein kinase A (PKA) via cyclic AMP. This is followed by three fundamental events: 1-release of fatty acids, 2-synthesis of the uncoupling protein1 (UCP1), 3-mitochondriogenesis [11]. The release of fatty acids causes activation of the mitochondrial UCP1 and activation of their mitochondrial oxidation, the other two activities are in direct relation and consequent to the thermogenic request. The oxidized molecules in the respiratory chain induce the formation of a proton gradient between the two mitochondrial compartments separated by the inner membrane. Normally the gradient is exploited by the ATPase which uses the proton flow to form ATP. In the brown adipocyte the UCP1, which is a protonophore, defeats the gradient and all that remains of the energy intrinsic to the molecules of oxidized fatty acids is the heat produced by oxidation as an inevitable secondary effect. Since the number of oxidized molecules is very high and the mitochondria that burn them are numerous, large and rich in cristae, the heat produced is functionally relevant [23]. BAT is able to allow the survival of mammals in areas of the planet where the environmental temperature is often below the thermoneutrality. If we consider that the body temperature must remain at 37 ° C and that the temperature in the Earth, where humans live, varies from +50 to -70 we can easily understand the need to have above all efficient thermogenic systems.

The Adipose Organ

Anatomical dissections have shown that most of the body's fatty tissues can be removed as a single structure from the body of small mammals (Fig. 2). The unitary structure, called Adipose Organ, has a specific shape that is maintained in different ages and sexes [4, 6, 24-27]. It is composed of parts arranged in the subcutaneous compartment and intra-trunk or visceral parts. In mice the subcutaneous part is particularly developed in correspondence of the roots of the limbs. The visceral part is arranged mainly around the aorta and its main collateral vessels. The continuity between the parts is achieved at the level of the upper opening of the thorax and in correspondence with the inferior narrow of the pelvis.

Macroscopically the color of the organ is brown in the parts containing BAT and white in the parts containing WAT. BAT prevails in the anterior subcutaneous depot at the interscapular, subscapular, deep cervical and axillary parts. In the visceral intra-trunk part BAT is prevalent in the periaortic areas. Throughout the rest of the organ, WAT prevails.

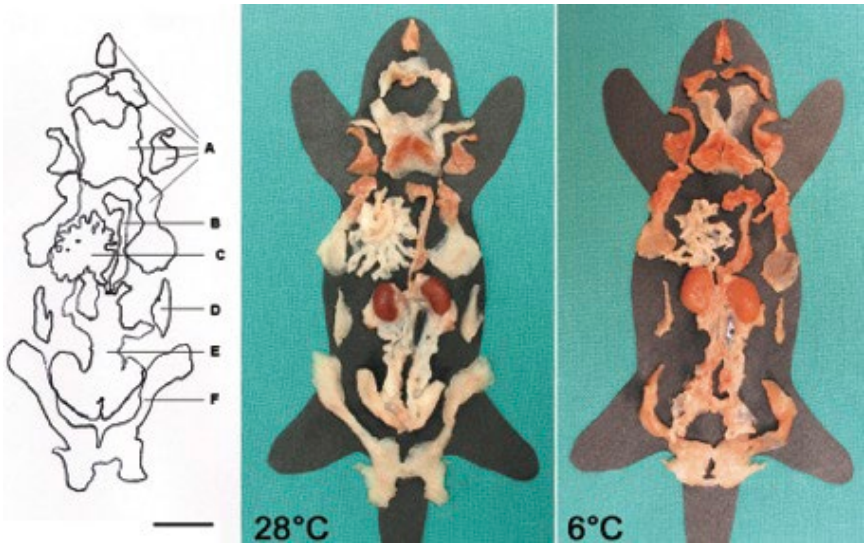

Fig 2. The adipose organ of adult Sv129 female mice kept at 28°C or 6°C for 10 days. The organ was dissected with the aid of a surgical microscope and each depot was placed on a mouse template indicating its original anatomical position. Kidneys and ovaries were dissected together with the depots. The organ is made up of two subcutaneous depots: A=anterior (deep cervical, superficial cervical, interscapular, subscapular, axillo-thoracic) and F= posterior (dorso-lumbar, inguinal, gluteal). Several visceral depots are also visible: B= mediastinal, C= mesenteric, D= retroperitoneal and E= abdomino-pelvic (perirenal, periovarian, parametrial, perivesical). Reproduced from [36] with permission. Bar: 18 mm.

The microscopic anatomy revealed that many white areas are actually mixed and morphometric studies have shown that in animals kept at 28°C (close to thermo-neutrality) in the strain Sv129 about 60% of the organ is formed by BAT while this percentage in the C57/BL6 drops to 20%.

Therefore, the adipose organ is mixed, i.e. it is formed by WAT and BAT which in some areas are clearly separated but which in some areas form a mixed tissue.

In humans, the study of digitized corpses has allowed us to reconstruct the human adipose organ that has many characteristics similar to those described above.

In particular there are parts located in the subcutaneous and visceral compartments with a histologic organization similar to the murine one. The predominant tissue in adult humans is WAT but areas of metabolically active BAT in the supraclavicular region of the neck have been demonstrated in adult humans. The technologies used for this demonstration were numerous including PET (Positron Emission Tomography) [28-31], MRI (Magnetic Resonance Imaging) [32-34], morphological, immunohistochemical and ultrastructural analysis [35]. In our study of about sixty patients, all patients under the age of 30 had immunoreactive BAT for UCP1, 20-30% of subjects aged 40-50 and rare subjects over 60 years were positive. Most positive subjects had low BMI (lean), but some overweight subjects were also positive. No obese subject was positive in line with data that showed a correlation between the presence of BAT and body weight [28].

Adipose organ plasticity

The minimum requirement to define an anatomically dissectible structure as an organ is that it is formed by at least two different tissues that cooperate finalistically between them. For example, the stomach is undoubtedly an anatomically dissectible organ in which different tissues cooperate for digestion. To identify the cooperation between WAT and BAT we investigated the organ in different functional conditions and we saw how exposure to cold determines a browning of the adipose organ (Fig. 2). This chromatic variation corresponds from the quantitative point of view to an increase in BAT with a corresponding equivalent reduction in WAT in the absence of any variation in the total number of fat cells that make up the organ [36]. This result was repeated and obtained in two different murine strains [36, 37]. Histological analysis has excluded apoptotic phenomena in line with the fact that the adrenergic stimulus protects the adipose cell from apoptosis. These data therefore favored an interpretation in line with previous experiments that suggested that WAT, subjected to adrenergic stimuli, transdifferentiates in BAT [38-40]. That is, the mature white fat cells, subjected to adrenergic stimulation, would be able to transform directly into brown fat cells [8, 41-45]. Detailed studies of these phenomena have shown that it is possible to demonstrate the presence of all the intermediate stages not only morphological but also of gene and protein expression. On the other hand, the total absence of the adrenergic stimulus induces the inverse phenomenon: the BAT converts to WAT. The ideal technique to definitively demonstrate the transition from one cytotype to another is that of lineage tracing. This technique is based on the fact that it is possible to indelibly mark a cytotype and detect the reporter gene even after the phenotypic conversion. Christian Wolfrum's group in Zurich used this technique to confirm our hypothesis [46, 47].

These data are particularly interesting due to the fact that BAT is essential to prevent obesity [48], T2 diabetes [49-51] and atherosclerosis [52]. In fact, genetically modified mice that cannot activate BAT because they lack the receptors necessary for its activation, while moving like controls and eating the same amount of food as the controls, in a few weeks they become massively obese [48]. Moreover, mice lacking the insulin receptor exclusively in BAT become diabetic [49] and BAT activation is able to prevent atherosclerosis [52] and prolong life [53]. The benefits of BAT activation have also been recently documented for humans [54].

All these data show that one can actually speak of an adipose organ also from a functional point of view, since the cooperation between WAT and BAT seems to be clearly demonstrated by their reciprocal conversion capacity to face particular functional needs of the organ. In particular the WAT-BAT conversion would serve to increase the organ's thermogenic properties in the event of chronic exposure to cold. Conversely, BAT-WAT conversion would serve to increase energy storage capacity in the event of chronic exposure to a positive energy balance. This implies three important consequences: 1-The need to verify whether white and brown fat cells have a common ancestor, 2-The possibility of establishing new therapeutic strategies to combat obesity, diabetes and atherosclerosis and to increase life expectancy stimulating the brown component of the adipose organ 3-Accepting a new cellular property: physiological and reversible transdifferentiation. The latter in fact implies that a mature cell can, physiologically and reversibly, modify its gene program and therefore change its phenotype and, consequently, its function.

The common ancestor of fat cells

In order to study the origin of adipose cells we observed the ultrastructure of two specific murine adipose depots during development: the epididymal for WAT and the interscapular for BAT. The choice of these two depots was dictated by the fact that in the adult animal the epididymal is exclusively made up of WAT whereas the interscapular by BAT therefore they represent the ideal depots to verify the origin of the white adipocyte and brown adipocyte respectively [1, 5].

The epididymal depot, before birth, appears as constituted by a mesenchymal tissue without specific characteristics. A few days after birth the tissue is organized to form specific, well-organized structures: the vasculo-adipocytic islands (Fig. 3). The name derives from the fact that these structures are well delimited by fibroblast-like elements that separate them from a loose connective matrix and contain numerous capillary vessels surrounded by fat cells in various stages of development. Intra insular tissue also contains numerous and dense collagen fibrils and to other cellular elements such as fibroblasts and mast cells. No adipocyte is found outside the islands, so they undoubtedly represent the niches of tissue where the progenitor cells give rise to the development of the preadipocytes destined then to form the adipocytes [55]. The vascular origin of the fat cells has been formulated for a long time and in particular the pericytes of the capillaries have been indicated by many researchers as a possible source for the preadipocytes [56, 57], but which was the cell that gave rise to the pericytes was not known.

The presence of a high number of capillaries in a tissue in the initial phase of differentiation, in itself underlines the possible instrumental value of these blood vessels, well represented not so much for the nutritional needs of the tissue but, probably, to guarantee the source of the precursors of the adipocytes. We therefore studied the ultrastructure of these capillaries for a long time, which proved to be fruitful because, in addition to the abundant pericytes, we also identified cells in a somewhat anomalous position that we have termed endothelium-pericytes. These rare elements (about 1/100 endothelial cells) were in fact positioned in the capillary wall in such a way as to place a part of the cell in an endothelial position and partly in a pericytic position. The endothelial position was demonstrated by contact with the lumen and by the typical tight flute beak junction with the contiguous endothelial cell. The pericytic position was demonstrated by the location of about half of the cell itself outside of that of an endothelial cell of the capillary (Fig. 4). Between it and the basement membrane of the capillary itself, that is to say in a classical pericytic position [55].

These ultrastructural data were suggestive of an endothelial origin of the pericyte, and given the unanimous consensus on the fact that the pericyte differentiate into adipocyte, it could be hypothesized that the endothelial cell represents the progenitor of the white adipose cell. To test this hypothesis, we used the lineage tracing technique. To mark the endothelial cells exclusively we used Ve-Cad-Cre/LoxP double transgenic mice already created and well characterized by the laboratory of Maria Luisa Iruela-Arispe of the Department of Molecular, Cell and Developmental Biology and Molecular Biology Institute, UCLA, Los Angeles, California [58]. These animals express the gene reporter (ß-Gal) only in endothelial cells and in any cytotypes derived from them. Our data confirmed that the gene reporter was expressed only in endothelial cells and showed that all fat cells in various stages of differentiation and

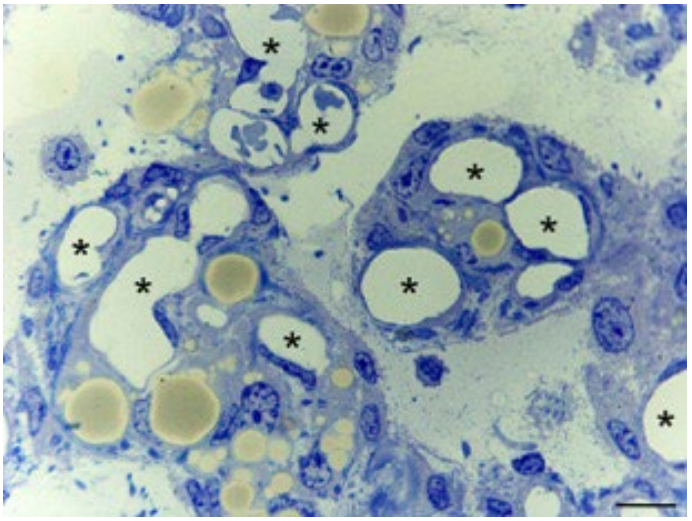

Fig 3. Vasculo-adipocytic islands in epididymal fat of newborn mouse. Structural units, delimited by fibroblast-like cells, functioning as anlage for white adipose tissue development. Note the numerous large capillaries that correspond to niches for adipose cell precursors development. Adipocytes at various stage of development are marked by cytoplasmic lipid droplets (yellow), note their anatomical localization only into the islands. Bar: 10 mm.

in various subcutaneous and visceral deposits were marked (Fig. 5). The X-Gal reaction that is used to highlight ß-Gal can also be exploited by electron microscopy [59]. In fact, the reaction product is not only visible in optical microscopy in the form of a cytoplasmic green-blue color but also as well-visible electron-dense crystals in electron microscopy. Electron microscopy confirmed the presence of reporter gene crystals in endothelial cells, fat cells and in all intermediate forms [55].

Interscapular BAT develops in a well-circumscribed area of the adipose organ. In the murine term fetus this area is characterized by the presence of brown preadipocytes. In Ve-Cad-Cre/LoxP mice this area was strongly marked by the reporter gene not only in endothelial cells but also in all brown preadipocytes. The differentiation level of brown preadipocytes allowed the specific identification of these cells as they are marked by the specific protein UCP1.

These data have therefore identified the endothelial cell of the adipose tissue capillaries as the sole progenitor for both white and brown cytotypes [55, 60]. This data allows us to understand even better the property that these cells have of converting themselves into each other.

A new example of physiological and reversible transdifferentiation

The physiological and reversible transdifferentiation as a new biological property of mature cells deserved further study and in particular the search for a new example. This example was provided by the mammary gland in the adipose organ. The

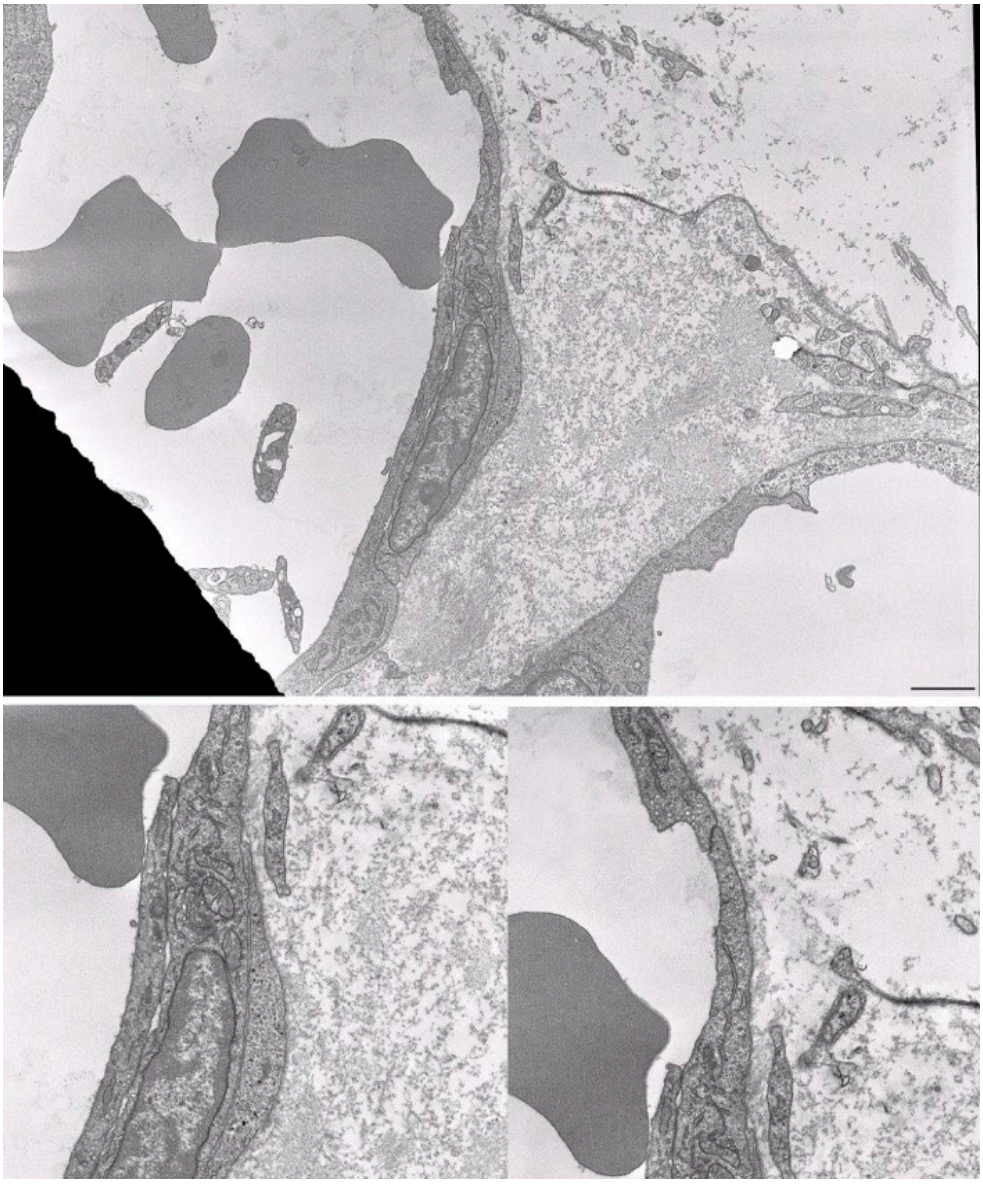

Fig 4. Transmission Electron Microscopy of a capillary shown in Fig 3. In the upper panel an endothelial cell is forming a cytoplasmic projection climbing on the adjacent endothelial cell. Note the presence of a cell in endothelial-pericytic position. The pericytic part is enlarged in the bottom left panel, the endothelial part is enlarged in the bottom right panel. Reproduced from [55] with permission. Bar: 1 mm in upper panel, 0.5 mm in bottom panels.

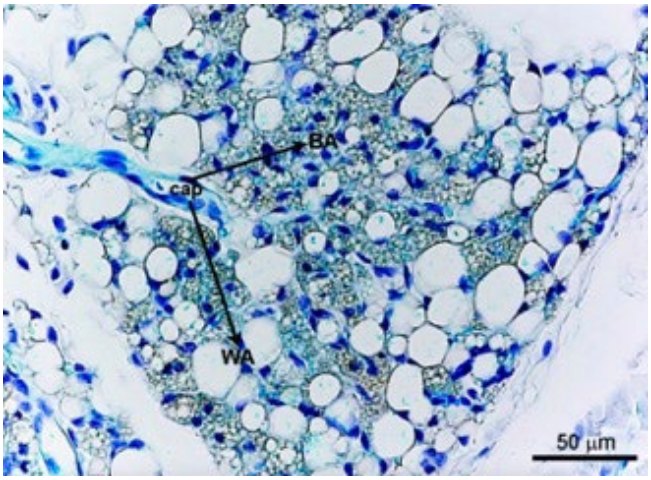

Fig 5. Subcutaneous fat from Ve-Cad-Cre/LoxP double transgenic mouse. X-Gal cytochemistry. In these mice, only endothelial cells and white (WA) and brown adipocytes BA) express the gene reporter (ß-Gal: green-blue). Reproduced from [60] with permission. Bar 50 mm.

mammary gland in the virgin is composed of adipose (predominantly white) subcutaneous tissue infiltrated by branched ducts that collect in a single nipple. The infiltration area identifies each individual gland and, in the mouse, the entire subcutaneous is infiltrated at puberty. In the mouse, the ducts refer to five symmetrical nipples, for which ten mammary glands are described. In women, only the thoracic subcutaneous is infiltrated and has two nipples.

During the pregnancy the alveoli appear, that is the adenomeres of the gland that produce the milk. They progressively occupy the organ volume while the adipose component in parallel disappears. At the height of breastfeeding, 90% of the organ is composed of dilated alveoli rich in milk and ducts, while fat cells are rarely visible (Fig. 6). At the end of breastfeeding, within a few days you return to the initial anatomy with disappearance of the alveoli and reappearance of the adipocytes [61].

A series of experiments carried out to evaluate these evident plasticity phenomena occurring in adult organisms allowed us to hypothesize that the basic phenomenon was an adipose-epithelial transdifferentiation in pregnancy and lactation and an epithelial-adipose transdifferentiation in post-lactation. We used aP2-Cre/LoxP mice to study the former and WAP-Cre / LoxP to study the latter. aP2 (adipocyte Protein 2) is a protein that acts as a transporter of fatty acids and is a specific gene for fat cells. Indeed, aP2-Cre/LoxP mice express the gene reporter only in adipose cells and in any cytotypes derived from them, while WAP-Cre/LoxP mice express the gene reporter only in milk-producing epithelial cells. In fact, Wey Acidic Protein (WAP) is a milk protein expressed only by breast alveolar cells during pregnancy and lactation.

These lineage tracing experiments confirmed the experimental hypothesis [62]. Moreover, transplantation experiments have further confirmed. In fact, both the transplantation of pure marked adipose tissue and that of mature, marked isolated mature adipocytes have shown the development of marked glands in pregnancy [63].

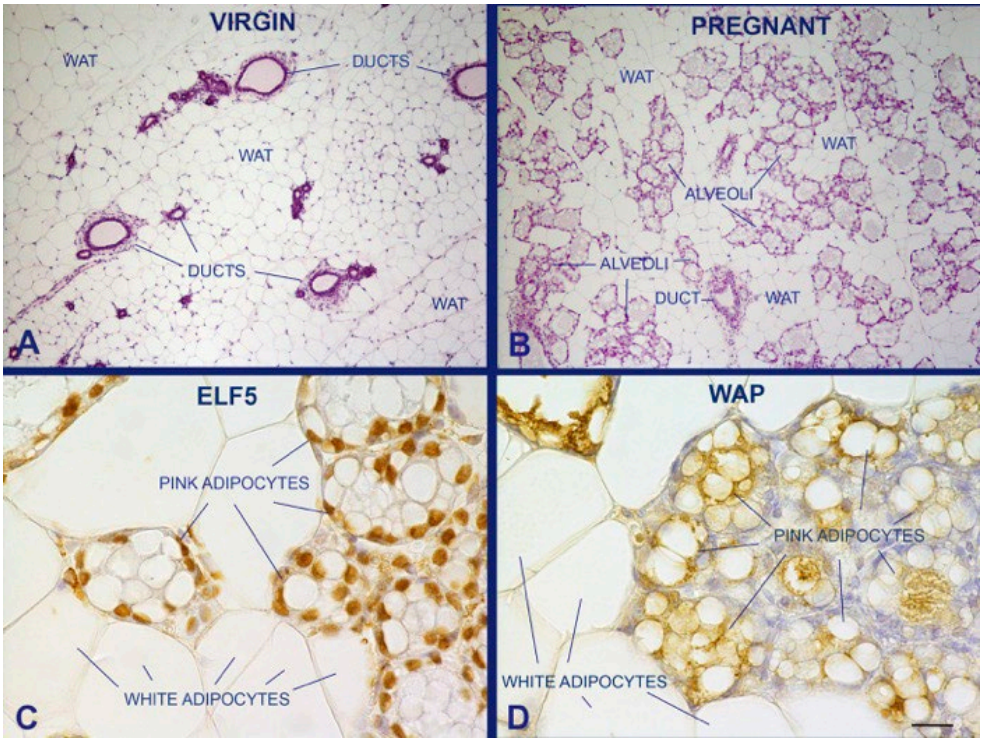

Fig 6. Histology of mammary gland of adult female mice. Alveoli composed by pink adipocytes are absent in virgin mice (A) and appear in the second half of pregnancy (B). Pink adipocytes (C and D) are immunoreactive for the master transcription factor of alveologenesis (ELF5) in nuclei (C) and for the milk protein WAP (whey acidic protein) in cytoplasm (D). Reproduced from [2] with permission. Bar: 50 mm in A and B, 12 mm in C and D.

In order to study the molecular mechanisms responsible for this tissue plasticity, we removed the ductal component monolaterally in animals that were subsequently pregnant.

The glands private of the ductal component (cleared fat pad) did not undergo any modification of the adipocytes during pregnancy, while the contralateral glands developed normally as described above. This result was somewhat expected because it is well known that the entire visceral component of the adipose organ does not respond to the hormonal pregnancy stimulus. In order to study the possible paracrine factor responsible for adipose-epithelial transdifferentiation, we performed a comparative analysis using microarrays between the cleared fat pad and the contralateral glands in mice at different stages of pregnancy. The data showed that osteopontin (Spp1) could be a candidate among the factors responsible for adipose-epithelial transdifferentiation [64-66]. It is in fact secreted by the ductal epithelium [67], its presence is necessary for normal alveologenesis [68, 69] and its transgenic overexpression causes alveolar hyperplasia [70]. We also highlighted the expression

of the master regulator of the ELF5 alveologenesis in the nucleus of adipocytes in the transdifferentiation phase, while the "normal" fat cells never expressed this transcription factor.

The new concept of nutritional system

In the organism of mammals, the organs work together for complex functions dedicated to maintaining the short and long-term homeostasis of the organism itself. In particular, the organs of the digestive system collaborate with the adipose organ for important homeostatic activities: the nutrition of the organism and of the offspring. For these complex functions it is necessary to influence the brain in that part which determines instinctual behavior. Both the adipose organ and the digestive system produce hormones that influence eating behavior. In fact, the organs of digestion produce Ghrelin, PYY^{3-36}, insulin, GLP1 which essentially act as orexigen (ghrelin) or anorectics (the others) on the hypothalamic centers [71-76]. The adipose organ produces leptin and asprosin (see above). Therefore, the adipose organ and organs of the digestive tract cooperate in activities related to the research and intake of food.

Furthermore, they produce hormones and substances (FGF21, secretin, bile acids) able to mutually modulate thermogenesis and this last influence, especially in newborns, the rhythm in food intake [77-81].

Finally, several data seem to support the idea that intestinal microbiota influence both gastrointestinal functional activity and adipose organ browning reinforcing the data supporting their functional relationships in this contest [82-92].

Therefore, the whole of the adipose organ and of the digestive system can be considered a system of the organism able to influence the fundamental behavior for survival (research and food intake), the absorption of nutrients (digestive system), the nutrient distribution to the entire organism (adipose organ) and to newborns (adipose organ temporarily transformed into a mammary gland).

Finally, considering all together, my proposal is therefore to add to the usually recognized systems of the human body, such as the nervous, cardiovascular, respiratory, endocrine, excretory, immunological and reproductive systems, a new important system that can be denominated as nutritional system.

Acknowledgments

I am grateful to all the Collaborators of my laboratory who faithfully and with great dedication have helped me in the last 43 years of work in Ancona and to all external Collaborators. In particular I acknowledge the President and Board of Italian Society of Anatomy and Histology for allowing me to hold, for the fourth time, an opening plenary lecture at our National Congress (Parma in 2018, previous: Messina 1988, Palermo 1998, Verona 2008). Finally, I thank the Members of our Scientific Society who have always inspired and encouraged me to continue in my morphological studies.

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Circulatory system

Comprehensive review of the superficial veins of the forearm from a historical, anatomical and clinical point of view

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Abstract

The superficial veins of the forearm are prone to possess different patterns of anastomosis. This is highly significant, as venipunctures in the upper limb are among the most performed procedures in the world and they often rely on the veins of the cubital fossa. In addition, the relationship of these veins to the cutaneous nerves are also prone to vary and are often uncertain. These veins are also manipulated in the creation of arteriovenous fistula for dialisis, which remains as the best choice of treatment for renal failure patients. Such fistulas are often performed on the wrist or the cubital fossa, with the cephalic vein or basilic vein. It is known that anatomical variations of the vessels and nerves on the cubital fossa may induce the professionals to error, and one of the most common complications of venipuncture are accidental nerve puncture, which can lead to paresthesia and pain. We aim to perform a comprehensive review of the venous arrangements of the cubital fossa and their clinical aspects, as well as of venipuncture from a historical perspective and of the complications of venipuncture and arteriovenous fistula from an anatomical point of view, with the purpose of compiling available data and help healthcare professionals to reduce puncture errors or arteriovenous fistula complications and improve patient care.

Keywords

Cubital fossa, venipuncture, history, anatomical variations, superficial veins.

Introduction

The superficial veins of the forearm are often used for venipuncture and subsequent drug infusions. Furthermore, they are also manipulated during the creation of arteriovenous fistulas in hemodialysis patients (Lee et al., 2015,Reyes II, 2016,Yammine, Erić, 2016).

These veins may acquire different anatomical arrangements and anastomosis, especially in the antecubital region. Furthermore, their relations with cutaneous nerves may also vary (Mikuni et al., 2013,Pires et al., 2018,Yamada et al., 2008,Yammine, Erić, 2016).

Despite being one of the most common and often performed procedures, venipunctures are invasive and often painful, thus requiring knowledge regarding the

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morphological and functional aspects of the venous vessels as well as their anatomical relationships (Fukuroku et al., 2016,Mikuni et al., 2013,Moore et al., 2014,Tsukuda et al., 2016).

In addition, the rates of venipuncture failure are often attributed to difficulty in assessing the veins of the forearm, and the quality of the venous access is directly related to the quality of the performed procedure (Ialongo, Bernardini, 2016,Lewis et al., 2013). Lack of knowledge may lead to repetitive attempts in cases where the veins are not prominent enough, which can lead to complications such as hemorrhage and phlebitis (Fukuroku et al., 2016,Kim et al., 2017)

In the field of arteriovenous fistulas, knowledge of the venous arrangements of the cubital fossa may imply better outcomes and lower complications rates of the procedure (Jindal et al., 2006,Quencer, Arici, 2015,Reyes II, 2016).

The review presented herein aims at recollecting historical, anatomical and clinical data regarding these superficial vessels with the purpose to improve knowledge in this area and help clinicians to make successful venipuncture procedures.

Review

History of the circulatory system and venipuncture

The study of the circulatory system began with Hippocrates and Galen, in ancient Greece. However, their dissections were performed in animals such as monkeys, pigs and dogs, and as a result their anatomical description lacked precision and was incorrect in several aspects (Goss, 1961,Persaud et al., 2014).

Despite their inaccurate descriptions, the superficial veins of the forearm were already being used centuries ago (especially the median antecubital vein) to draw blood by means of bloodletting and phlebotomy. These procedures were often used as a treatment for several conditions, e. g. seizures and fever (Greenstone, 2010,Parapia, 2008).

The median antecubital vein was often the site for bloodletting due to its relation with the bicipital aponeurosis: it prevented the physician to accidentally cut the brachial artery (Testut, Latarjet, 1958). Due to this property, some authors referred to the bicipital aponeurosis as the "fascia of the grace of God" (Burdan et al., 2016,Lacombe, 1988,Moore et al., 2014).

In the XVII century, anatomists such as Sylvius and Vesalius revolutionized the anatomical world with their descriptions based on human dissections (King, 1970,Persaud et al., 2014).

Intravenous therapies were not studied until the XIV century, although the focus was placed in blood transfusion therapy. The first documented use of blood transfusion was in Rome (1492). The Pope Innocent VIII was in a coma and his physician thought that blood transfusion would cure him, thus, he obtained blood from three healthy young men and started the therapy. Not long after, the three men and the Pope died (Rivera et al., 2005).

After this tragic event, intravenous infusions weren't the focus of research until Williiam Harvey's description of the circulatory system in his book *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus*". Harvey described not only the sys-

temic and pulmonary circulation, but the venous valves as well. Thus, he enhanced knowledge regarding the cardiovascular system (Mandarim-de-Lacerda, 1990,Payne et al., 1998).

During this period, there was a tragic yet important event involving a servant, which was unjustly accused of infanticide (her baby was stillborn) and sentenced to death by hanging. With the purpose of speeding her death, she asked her friends to beating her up whilst hanging. Her cadaver was obtained by the anatomy laboratory of the Oxford University, but she was still alive, albeit with a faint pulse and weak breathing. This event severely impacted the students and teachers on the scene and inspired Christopher Wren and Richard Lower to develop instruments for intravenous infusions and make research on this procedure (Felts, 2000,Hughes, 1982,Rivera et al., 2005).

Concomitantly, there were several attempts to inject opium into the veins of dogs, although they yielded poor results due to the lack of specialized instruments and lack of knowledge regarding blood coagulation (Felts, 2000,Rivera et al., 2005)

The first real form of intravenous therapy was during the cholera outbreak in Europe (1831), due to poor sanitary conditions. Cholera is known to be a disease that causes symptoms such as diarrhea and vomit, thus, there is the need to maintain the hydric balance. William Brooke O'Shaughnessy deduced this hypothesis in 1832 and together with Thomas Latta – his student – they successfully injected (continuously) a saline solution in the basilic vein of a cholera patient, whom survived the disease (Jackson, 2003,Peterfreund, Philip, 2013,Rivera et al., 2005).

With the creation of the hollow needle in 1845 by Rynd (he wanted to intravenously administrate morphine to treat trigeminal neuralgia), with the advent of the hypodermic needle created by Charles Pravaz in 1853 and with the fabrication of the glass syringe devised by Georg Luer in 1869, intravenous therapies began to evolve and became more common among healthcare professionals (Duque, Chagas, 2009,Rivera et al., 2005)

Nowadays, venipuncture and intravenous infusion are among the most performed procedures in the world. It is important to notice that the evolution of venipuncture was paced together with more complex understanding of the morphology and physiology of the venous system, the creation of newer devices properly created for specific types of treatment and infusion demand/flow as well as newer mechanisms to provide and improve the quality and safety of this procedure (Lewis et al., 2013,Strauss et al., 2008,Zarychanski et al., 2009)

Anatomy of the superficial veins of the forearm

The superficial veins are situated between the two layers of the superficial fascia and are accompanied by cutaneous nerves and superficial lymphatic vessels. They usually possess valves and communicate with the deep venous system by the perforating veins (valveless vessels) (Gardner et al., 1978,Goss, 1977,Latarjet, Liard, 1993,Moore et al., 2014,Testut, Jacob, 1952,Testut, Latarjet, 1958).

Three great veins can be observed on the forearm: the basilic vein (BV), which arises from the medial side; the cephalic vein (CV), which arises from the lateral side; and the median antebrachial vein, which arises between these two vessels. The CV and the BV are product of the dorsal venous network of the hand, while the medi-

an antebrachial vein is originated by the small veins from the palmar surface of the hand (Goss, 1977,Testut, Jacob, 1952,Testut, Latarjet, 1958).

As they reach the antecubital fossa, these veins acquire different forms of anastomosis (Pires et al., 2018,Yammine, Erić, 2016). The description here is a review of what is described in anatomical textbooks, while their arrangements and variations are addressed further in the text.

Classically, several authors describe and depict an arrangement in which the median antebrachial vein joins the median cubital vein – a vessel that rises from the CV and flows into the BV – or in the absence of the median cubital vein, the median antebrachial vein bifurcates and originates the median basilic vein and the median cephalic vein, joining the BV and the CV respectively. The latter arrangement is known as the "Classic M" or Y-shaped (Goss, 1977,Testut, Jacob, 1952,Testut, Latarjet, 1958).

It is important to remind that the median basilic vein and the median cephalic vein were removed from the "Terminologia Anatomica" (Del Sol et al., 2007), however these names are still used in research articles (Corzo Gómez et al., 2010,Del Sol et al., 2007,Yammine, Erić, 2016) and new anatomical textbooks (Moore et al., 2014) as they are fundamental to understand the venous patterns.

There is mention in the literature regarding the accessory cephalic vein as well. This vessel originates from a smaller plexus on the dorsal aspect of the forearm, the medial end of the dorsal venous network and joins the CV. Furthermore, it can also originate from a more distal portion of the CV. (Goss, 1977,Testut, Jacob, 1952,Testut, Latarjet, 1958).

According to Gardner et al. [20]), the superficial veins of the forearm are the main channels for the venous return. This is accordance with Testut, Latarjet [62](1958), as the authors explain that these veins are remnants of the upper limb bud development and that during ontogenesis the deep veins ran towards them.

In addition, the superficial veins of the forearm are larger than the deep ones and are subjected to the actions of the muscles in the arm and forearm (Testut, Latarjet, 1958).

The cubital fossa and the venous arrangements of the superficial veins

The cubital (or antecubital) fossa is a triangular space with its apex pointed distally to the limb. Its lateral and inferior boundary is the brachioradial muscle, while the pronator teres muscle binds the cubital fossa medially and inferiorly. The base of the "triangle" is done by an imaginary horizontal line between both epicondyles of the humerus. Its floor is formed by the brachial and the supinator muscles, while its roof is composed by the superficial fascia, subcutaneous tissue and the skin (Ellis, 2010,Pires et al., 2018,Testut, Jacob, 1952).

The deep content of the cubital fossa includes the distal biceps tendon, the brachial artery, the median and the radial nerves, while the superficial content includes the CV, BV, median cubital vein and the lateral and medial cutaneous nerves of the forearm. These structures are separated by the bicipital aponeurosis (Ellis, 2010,Moore et al., 2014,Testut, Jacob, 1952). As previously stated, these elements - specially the venous ones - are prone to vary in this region. Furthermore, their anatomical relationships with other structures are subject to variation (Corzo Gómez et al., 2010,Del Sol et al., 2012,Pires et al., 2018,Yamada et al., 2008,Yammine, Erić, 2016).

First, we will describe the several patterns reported in the literature and briefly comment anatomical variations of other elements present in the cubital fossa.

The venous arrangement of the cubital fossa has been studied by several authors, and some have shown regional, side and gender differences among their populations (AlBustami et al., 2014,Berry, Newton, 1908,Charles, 1932,Corzo Gómez et al., 2010,Del Sol et al., 2007,Del Sol et al., 2012,Doyle, 1968,Hamzah et al., 2014,Okamoto, 1922,Ukoha et al., 2013,Yamada et al., 2008,Yammine, Erić, 2016). However, their comparison is difficult due to differences in methodology and numerous distinct classifications of venous anastomoses (Pires et al., 2018).

For instance, the "Type 1" (Figure 1) according to Yammine, Erić [68](2016) is the classic venous arrangement shaped like the letter M or Y. This type was the second commonest, according to their paper.

This pattern was described as type A (Ukoha et al., 2013,Wasfi et al., 1986), 2 (AlBustami et al., 2014), 3 (Charles, 1932,Hamzah et al., 2014), 3-B (Halim, Abdi, 1974), 5 (Corzo Gómez et al., 2010), and some authors classified it as type 1 (Lee et al., 2015,Singh et al., 1982,Yamada et al., 2008). When these studies are further analyzed, it can be seen that some authors only describe the M pattern when the accessory cephalic vein is present (Del Sol et al., 2007), which characterizes the seventh type according to the classification proposed by Yammine, Erić [68](2016).

There is also confusion among anatomical textbooks. As previously observed, in Gray's Anatomy textbook the author assumes that the M type is the same as the Y

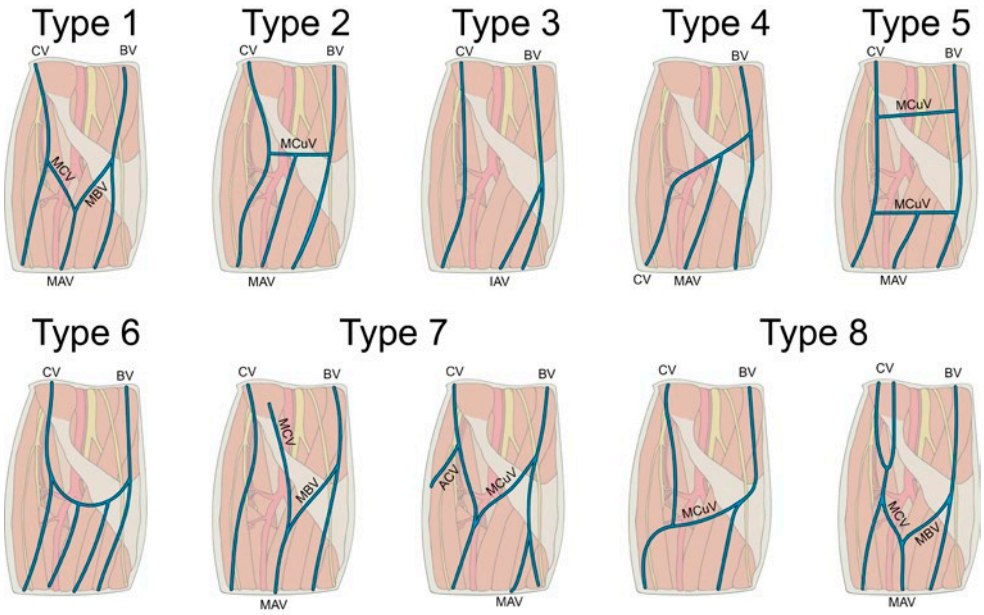

Figure 1. The venous patterns of the cubital fossa. CV = cephalic vein; BV = basilic vein; MAV = median antebrachial vein; MCuV = median antecubital vein; MBV = median basilic vein; MCV = median cephalic vein; ACV = accessory cephalic vein.

type, due to the disposition of the median antebrachial vein (Goss, 1977), despite that Sobotta's anatomical atlas refers to the Y shaped type as the one in which the CV joins the BV and there is only a rudimentary vein running towards the arm in the place of the CV (Jasiński, Poradnik, 2003,Sobotta, 2013).

Furthermore, Gardner et al. (1978) illustrate a pattern in which the accessory cephalic vein replaces the CV on the arm, although no such pattern has been described in the literature and it is not mentioned in the textual part of that book. There was only description of a similar pattern in a paper by Halim, Abdi [25](1974) in which, instead of the accessory cephalic vein, a deep vein from the cubital fossa replaced the CV in the arm, however no other paper describes this situations.

In order to avoid confusion, we propose that future works adopts the classification by Yammine, Erić [68](2016) as it is the most complete and versatile. The types are resumed in Table 1 and are depicted in Figure 1.

Type 2 was the most prevalent (44%-60%), followed by type 1 (20%-25%), type 7 (roughly 13%), type 3 (4%-11%), type 8 (roughly 8%), type 6 (approximately 4.5%), type 4 (3%-4%), and type 5 (approximately 2.4%) (Yammine, Erić, 2016).

The CV is not prone to vary in the arm and axilla, although it can be absent or rudimentary and sometimes it can completely join the external jugular vein or communicate with it (Araujo et al., 2017,Bergman et al., 1988,Loukas et al., 2008). A rudimentary state, but not absence of the BV in the arm has been described (Tubbs et al., 2016).

Variations of the brachial artery in the cubital fossa include its early division into radial and ulnar artery (Testut, Jacob, 1952,Testut, Latarjet, 1958). Its high division can produce the variations known as the superficial ulnar artery or the superficial radial artery (although this variant is rarer). These arteries are located on the superficial fascia, together with the superficial veins and the cutaneous nerves (Gruber, 1867,Narayanan, Murugan, 2017,Testut, Jacob, 1952,Testut, Latarjet, 1958,Tubbs et al., 2016). The bicipital aponeurosis (*lacertus fibrosus*) may be absent (Tubbs et al., 2016).

The cutaneous nerves in the cubital fossa also present different dispositions in respect to the superficial veins (Mikuni et al., 2013,Yamada et al., 2008). The lateral cutaneous nerve of the forearm is a branch of the musculocutaneous nerve, while the medial cutaneous nerve of the forearm comes directly from the medial cord of the brachial plexus (Goss, 1977,Latarjet, Liard, 1993,Testut, Latarjet, 1958). These nerves are often situated above the superficial veins and are more frequently present on the medial side of the cubital fossa (Mikuni et al., 2013,Yamada et al., 2008).

In the next section we will describe how all of these variations affect the procedures performed in the cubital fossa.

Clinical significance

The superficial veins of the forearm are commonly used in the clinical practice for venipuncture and arteriovenous fistulas for hemodialysis patients, as they are easy to reach and drain a significant portion of the upper limb blood (Elamurugan, Hemachandar, 2017,Gardner et al., 1978,Kumar et al., 2007,Lewis et al., 2013,Reyes II, 2016,Testut, Latarjet, 1958).

Superficial venous access is extremely relevant for daily clinical practice, as it can be used for numerous purposes, such as drug infusion, contrast injection to perform

several exams, and collection of blood samples (Ialongo, Bernardini, 2016,Lewis et al., 2013).

Complications of venipuncture can involve the aforementioned anatomical variations. Cutaneous nerve injury and accidental arterial cannulation have been reported due to the spatial disposition of the cutaneous nerves and the variant known as superficial ulnar artery, respectively (Ramos, 2014,Shivappagoudar, George, 2013).

We remind that iatrogenic drug infusion in arterial vessels may produce an extremely painful sensation which can develop to ulceration. This condition is known as *embolia cutis medicamentosa* or Nicolau syndrome (Chagas et al., 2016,Duque, Chagas, 2009). Accidental arterial puncture may also produce aneurysms or pseudoaneurysms (Leite et al., 2017).

The accidental puncture of cutaneous nerves can produce acute sharp pain (electrical-type pain) and paresthesia on the territory of the afflicted nerve. This can happen either during the moment of the puncture or several hours later. These symptoms often disappear on their own, although several patients have to seek medical help to relieve the symptoms (Kim et al., 2017,Ramos, 2014,Tsukuda et al., 2016). It is stated that most of these injuries involve the lateral cutaneous nerve of the forearm (Ialongo, Bernardini, 2016).

In addition, venipuncture possesses high failure rates (up to 30%), and often requires multiple attempts. Factors such as lack of skill, difficulty in assessing and observing the superficial veins or the age of the patients often compromise the success of this procedure. Repetitive attempts can often lead to high extravasation rate, hematoma, hemorrhage and phlebitis (Fukuroku et al., 2016,Ialongo, Bernardini, 2016,Kim et al., 2017).

Anatomical studies observed that the most dangerous region is near the medial margin of the antecubital fossa, due to the presence of more sensorial branches, and the safer zone would be the radial part of the antecubital fossa (Mikuni et al., 2013,Yamada et al., 2008). According to anatomical textbooks the most used vein for venipuncture is the median cubital vein, as it is large and more prominent (Gardner et al., 1978,Moore et al., 2014). The radial portion of the median cubital vein is the safest region to perform venipuncture (Yammine, Erić, 2016).

With the purpose of reducing iatrogenic errors during venipuncture, several institutions have adopted the new vein visualization display system, which works through near-infrared light and accurately displays the superficial venous network. Thus, it can be used as a guide during venipuncture procedures (Fukuroku et al., 2016,Lee et al., 2015).

Variations of the CV can interfere with peripheral insertion of a central catheter, in which the CV is punctured near the cubital fossa and a catheter is placed near the right atrium. As such, knowledge of its absence, rudimentary state and anastomoses become highly significant (Araujo et al., 2017,Chopra et al., 2014). Furthermore, the absence of the CV associated with venous compression on the medial end of the forearm or axilla can lead to cyanosis and swelling of the limb (Imanishi et al., 1998).

Arteriovenous fistula is the most frequently performed procedure for dialysis. It is recommended nearly in all cases due to lower rates of failure, infection, steal syndrome and thrombosis, as well as higher lifespan in comparison to other treatments (Quencer, Arici, 2015).

The preferred sites for AVF creation are the wrist (radial artery and CV) and

elbow (brachial artery and CV), although the BV can be used as well. The use of lower limb veins is not recommended. Despite that, each patient possess unique characteristics, thus, eligible sites for AVF creation are often individualized (Jindal et al., 2006,Kumar et al., 2007). The median antebrachial vein or the median basilic vein can be used in cases in which the CV was rudimentary or had a small caliber (Elamurugan, Hemachandar, 2017).

Evaluation of the caliber, length and possible obstruction sites of the vein and artery is necessary. Complications such as aneurysm formation, thrombosis and steal syndrome are highly dependent on the quality of the used vessels (Jindal et al., 2006).

Moreover, the superficial veins of the upper limb can be sites of aneurysms (Faraj et al., 2007,Katsoulis et al., 2003,Yaylak et al., 2007) and superficial vein thrombosis (Cosmi, 2015), which further makes the study of these vessels relevant.

Conflicts of interest

The authors have no conflict of interest to declare.

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Circulatory system

Renal artery variations: a 20.782 kidneys review

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Abstract

Introduction: vascular anatomy variations are important in academic, clinical and surgical areas as well as nephrology, urology, oncological and vascular surgery, among others. The main objective of this review is to know the real prevalence of multiple renal arteries in a wide, multiethnic population. Secondary objectives are to establish the prevalence of early branching of the renal artery and the prevalence of these variations in left and right kidneys. **Methods:** this study analyzes the renal arterial anatomy of 20.782 kidneys from 64 anatomical and radiological studies. **Results:** Multiple renal arteries (MRA) were present in 19,95% of the total kidneys, in number of 2 to 6 arteries arriving to the hilum. The most frequent number of MRA was 2 renal arteries (89,48%), followed by 3 (9,31%), 4 (1,06%), 5 (0,02%) and 6 (0,005%). This last one being found in only one kidney. Reported data on the lateralization of the MRA are rather poor, and among these no side's predilection was found: MRA were found in 49,83% on the right side and 50,17% of left kidneys. Early branching patterns were described in only one third of the published data, being present in 11,4% of the total kidneys from those data (corresponding in 4,23% of right kidney cases and in 4,52% of left kidney cases; 2,66% had no right/left information). **Discussion:** the most difficult part was to merge the results from the different studies due to the heterogeneity of their descriptions. A universally accepted medical nomenclature is needed in order to allow a more precise lecture and transmission of results in clinical practice. Renal anatomical variations have clinical and surgical implications in renal transplantation, correctable hydronephrosis, ablation treatment for refractory hypertension or endovascular reconstructions and should be taken into account by every physician.

Keywords

Renal artery branching pattern, renal vascular anatomy, kidney arterial blood supply, renal artery variations.

Introduction

Several attempts to study the frequency of occurrence of renal vascular variations have been made, being the first one that from the Anatomical Society of Great Britain and Ireland in 1891 (Thomson et al., 1889). Most of the works published up to now included small groups or narrowed ethnicities. The present article reviews more than 20.000 kidneys of people from 31 countries on the five continents. To our knowledge is the most extensive and varied review ever performed.

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The continuously changing blood supply of the kidneys as they ascend from the pelvis to their final location during the embryological development explains the high incidence of anatomical variations.

The first description of accessory renal arteries was given in 1564 by Eustachi, and since then renal artery terminology has been controversial and unclear (Gulas et al., 2018). Nowadays there is no universally accepted nomenclature to describe arteries arriving to the kidneys other than the "normal main renal artery". Additional renal arteries have been variously described as accessory, aberrant, anomalous, supernumerary, supplementary, multiple and many other terms (Satyapal et al., 2001).

Authors like Satyapal et al. propose and use the following definition: an additional renal artery, other than the main renal artery, is one which arises from the aorta and terminates in the kidney. Other authors like Klatte et al.(2015) referred as accessory artery to any supernumerary artery that reaches the kidney. If the artery does not enter the kidney at the hilum, it is called as aberrant .

Bergman et al. (2018) said that additional renal arteries, varying in size and generally derived from the aorta, are found in 26 to 30% of kidneys entering at almost any point of the parenchyma. They sustain it is a misnomer to call such vessels accessory renal arteries because they are not extra but essential, tissue-sustaining arteries and cannot be bound without consequences.

Variations on the renal arteries not only refers to the number, but also to the division point. For example, Sampaio and Passos (1992) "limited" the early division to 1 cm from the aorta, whereas Saldarriaga et al. (2008) "expanded" this distance to 2 cm, with profound differences in their results.

Due to the multiple definitions found in literature, a consensus between the authors allowed to define an accessory renal artery as any branch from the aorta or other main arteries that enters the kidney through the hilum, including those arteries born from the division of the main renal artery into different branches. The polar arteries were defined as those arteries that have their origin in the aorta or other main arteries different from the renal artery that enter the kidney through the upper or the lower pole.

The occurrence of accessory renal arteries may be concomitant not only to other atypical variants of vascularization, especially those of the genitourinary system, but also to some specific medical conditions (Anjamrooz et al., 2013).

Precise knowledge of the most frequent types of renal vascular anatomical variations is needed due to the continuous growing and development of surgical procedures such as renal transplants, aneurysms repair, oncologic partial nephrectomy, ablation treatment for refractory hypertension and vascular reconstructions (Gulas et al., 2018; Klatte et al., 2015; Aldana et al., 2010; Urban et al., 2001; Benedetti et al., 1995; Ali-El-Dein et al., 2003; Abbasi et al., 2011; Mendes et al., 2016).

In any surgical technique, laparoscopic or classical open approach, unawareness of the presence of an additional renal artery may result in a fatal outcome and many studies tried to understand the real impact of these anatomical variations.

The main objective of this review is to know the real prevalence of multiple renal arteries in a wide, multiethnic population. The secondary objectives are to establish the prevalence of early branching of the renal artery and the prevalence of these variations in left and right kidneys.

Materials and methods

During the pre-analytical stage, we performed a wide range research. Classic research databases such as Medline, Embase, and Web of Science databases were screened. Also other well known online libraries such as Scientific Electronic Library Online (www.scielo.org) and Europe Pubmed Central (www.europepmc.org) were screened. Due to the low number of cases found, we decided to extend the search into several Anatomy and Morphology sciences journals, such as Scholar Science Journals (www.ssjournals.com), Hindawi Publishing Corporation (www.hindawi.com), International Journals in Medical and Health Research (www.ijmhr.com), International Journal of Experimental and Clinical Anatomy (www.anatomy.org.tr), Romanian Journal of Morphology and Embryology (www.rjme.ro), African Journals Online (www.ajol.info), Asian Pacific Journal of Health Sciences (www.apjhs.com), Firenze University Press (www.fupress.net), American Journal of Roentgenology (www.ajronline.org), Via Medica Journals (www.journals.viamedica.pl), Impact Journals (www.impactjournals.us), Revista Argentina de Anatomía Clínica (www.anatclinar.com.ar).

Database research was performed without time limit using the keywords: "renal artery branching pattern", "renal vascular anatomy", "kidney arterial blood supply", "renal artery variations", "variaciones arteria renal", "vascularización renal", "variação arteria renal", "variantes anatómicas arterias renales".

The search was not limited to the English literature because authors are native speakers or have a bilingual level in languages such as Spanish, Greek, Italian, English, Portuguese, French or Romanian, and so literature analysis and/or interpretation would not be affected. Another reason that led us to include these articles was the fact that they would add not only a significant number of cases but also a biological diversity to this work, being the last one an invaluable characteristic in order to extrapolate the results to general population.

One hundred and thirty two articles passed the pre-selection. In a second stage, repeated articles or articles with incomplete information were discarded. Eighty articles were judged to be highly relevant by the individual authors and were circulated among the rest of the working group. A total of 20.782 kidneys were included in the present work.

Results

We have analyzed 64 articles with a total of 20.782 kidneys. Multiple renal arteries (MRA) were founded in 4.146 kidneys (19,95%) of the total. The number of MRA arriving to the kidney varied from two to six (Thomson et al., 1889; Satyapal et al., 2001; Sampaio et al., 1992; Saldarriaga et al., 2008; Ali-El-Dein et al., 2003; Katariya et al., 2015; Bordei et al., 2004; Raman et al., 2007; Çiçekcibaşi et al., 2005; Hung et al., 2012; Harrison et al., 1978; Ugurel et al., 2010; Kaneko et al., 2008; Virendra et al., 2010; Budhiraja, et al., 2013; Özkan et al., 2006; Natsis et al., 2014; Bouali et al., 2012; Stanca et al., 2009; Jacek et al., 2007; Kornafel et al., 2010; Aristotle et al., 2013; Tarzamni et al., 2008; Méndez López et al., 2014; Sofía et al., 2008; Olave et al., 2009; Shaikh et al., 2014; Vasi et al., 2015; Palmieri et al., 2011; Ayuso et al., 2006; Patil et al., 2001; Jee et al., 2008; Platt et al., 1997; Johnson et al., 2013; Soares et al., 2013; Talović

et al., 2007; Munnusamy et al., 2016; Zağyapan et al., 2009; Saritha et al., 2013; Vatsala et al., 2014; Zăhoi et al., 2015; Tayyba et al., 2016; Refaat et al., 2013; Aragão et al., 2012; Calle Toro et al., 2016; Khamanarong et al., 2004; Aubert et al., 1975; Holden et al., 2005; Janschek et al., 2004; Costa et al., 2011; Tyson et al., 2011; Kapoor et al., 2011; Tao et al., 2013; Kok et al., 2008; Han et al., 1998; Chabchoub et al., 2011; Sezer et al., 2012; Lloyd et al., 1935; Vilhova et al., 2001; Gümüş et al., 2012; Mustafa et al., 2016; Lawton et al., 2017; Cases et al., 2017).

Between those 4.146 kidneys, 3.710 (89,48%) had 2 arteries, 386 (9,31%) had 3 arteries, 44 kidneys had 4 arteries (1,06%), 5 kidneys had a total of 5 arteries (0,02%) and only 1 case described a kidney with 6 renal arteries (0,005%). Table 1 resumes these findings.

Only 34 of the articles made the distinction between right and left kidney with MRA. Among the 12.446 kidneys included in those articles, 2308 had MRA distributed 1150 (49,83%) in right kidneys and 1158 (50,17%) in left kidneys (Table 2) (Satyapal et al., 2001; Saldarriaga et al., 2008; Katariya et al., 2015; Bordei et al., 2004; Raman et al., 2007; Harrison et al., 1978; Ugurel et al., 2010; Kaneko et al., 2008; Özkan et al., 2006; Natsis et al., 2014; Bouali et al., 2012; Kornafel et al., 2010; Shaikh et al., 2014; Vasi et al., 2015; Ayuso et al., 2006; Patil et al., 2001; Johnson et al., 2013; Soares et al., 2013; Talović et al., 2007; Zağyapan et al., 2009; Saritha et al., 2013; Refaat et al., 2013; Aragão et al., 2012; Calle Toro et al., 2016; Khamanarong et al., 2004; Aubert et al., 1975; Holden et al., 2005; Costa et al., 2011; Tyson et al., 2011; Tao et al., 2013; Gümüş et al., 2012; Mustafa et al., 2016; Sungura et al., 2012).

Early branching was described in as few as 21 of the 64 articles, but no universal definition was used. Some of them defined early branching using an arbitrary limit of 1,5 or 2 cm between the aorta and the distance within the division of the main renal artery in MRA, while some others did not specify a limit. Between the 8769 kidneys described in those articles, 1000 (11,4%) presented an early branching (all definitions

Table 1. Number of kidneys with MRA and its prevalence.

	Number of MRA described					
	Two (%)	**Three (%)**	**Four (%)**	**Five (%)**	**Six (%)**	**Total (%)**
	3710 (89,48)	386 (9,31)	44 (1,06)	5 (0,12)	1 (0,02)	4146 (100)
% from the 20782 kidneys	17,85	1,86	0,21	0,02	0,01	19,95

Table 2. Prevalence of MRA in right and left kidneys.

	Number of MRA (%)				
	Two	**Three**	**Four**	**Five**	**Total (%)**
Right kidneys number (%)	1043 (45,19)	93 (4,03)	12 (0,52)	2 (0,09)	1150 (49,83)
Left kidneys number (%)	1055 (45,71)	87 (3,77)	14 (0,61)	2 (0,09)	1158 (50,17)
					2308 (100)

Table 3. Early branching prevalence.

	Early branching side			
Total Kidneys (%)	**Right (%)**	**Left (%)**	**Not specified**	**Total (%)**
8769 (100)	371 (4,23)	396 (4,52)	233 (2,66)	1000 (11,40)

considered together). Only a minority of the articles included lateralization information of the early branching: 371 (4,23%) were right kidneys and 396 (4,52%) were left kidneys. In 233 (2,66%) of the cases no right/left side was specified (Table 3) (Thomson et al., 1889; Sampaio et al., 1992; Raman et al., 2007; Hung et al., 2012; Budhiraja, et al., 2013; Özkan et al., 2006; Jacek et al., 2007; Kornafel et al., 2010; Tarzamni et al., 2008; Ayuso et al., 2006; Patil et al., 2001; Jee et al., 2008; Platt et al., 1997; Munnusamy et al., 2016; Tayyba et al., 2016; Refaat et al., 2013; Holden et al., 2005; Tao et al., 2013; Kok et al., 2008; Gümüş et al., 2012; Mustafa et al., 2016).

Discussion

Theoretically, a higher risk of surgical complications to the living donor and to the recipient is associated to the renal graft with accessory arteries. In the past, its presence was considered as a contraindication to the procedure, but nowadays most of the studies agree that MRA are not considered a problem anymore neither to open nor to laparoscopic nephrectomies (Benedetti et al., 1995; Ali-El-Dein et al., 2003; Abbasi et al., 2011; Mazzucchi et al., 2005; Carter et al., 2005). Some others still consider its presence as a contraindication to their use. They justify their position saying that as these are end-arteries they should all be re-implanted requiring several anastomoses with a prolonged ischemic time, leading to a theoretically higher incidence of renal failure, graft rejection, marked decrease in renographic clearance post-transplantation, and reduced graft function (Ashraf et al., 2013; Harraz et al., 2013).

Additional renal arteries might also condition aortic aneurysm endovascular repair, not only because of their presence, but also because of the amount of parenchyma perfused by them. According to Mendes et al. (2016), there is no consensus on how much renal parenchyma can be safely sacrificed during endovascular aortic repair. While some authors have proposed that sacrificing an entire kidney to seal the aneurysm is acceptable in difficult case (Satyapal et al., 2001), an arbitrary exclusion criteria for endovascular repair in fenestrated-branched devices trials was proposed by Mendes et al., they chose an open repair technique or other alternative treatment when more than 40% of one kidney or 25% of both kidneys need to be sacrificed to accomplish endovascular incorporation.

Renovascular hypertension is a form of secondary hypertension typically caused by atherosclerosis or fibromuscular dysplasia, but also by ureteropelvic junction obstruction caused by functional or anatomic anomalies. The extrinsic compression

of the ureteropelvic junction by an accessory renal artery can be found among the causes (Lee et al., 2015). Even if rare, this is a surgically reversible cause of secondary hypertension that should be researched and treated.

In a study of patients with resistant hypertension and their response to arterial denervation performed by Verloop et al. (2014), 34% of the patients had accessory renal arteries. Their results suggest that renal denervation is effective in patients with multiple renal vessels, especially in those patients with arteries which could all be treated. Due to these results and the high prevalence of MRA among patients with resistant hypertension, they concluded that it seems reasonable not to exclude patients with accessory arteries from this treatment .

Accessory renal arteries are also determinant in oncologic conservative surgery techniques. The incidence of renal tumors has increased over the past several decades. Most of the patients are diagnosed at clinical stage T1, making them electable to partial nephrectomy. Nowadays minimally invasive partial nephrectomy has become an alternative to open surgery, a detailed understanding of surgical anatomy is basic to optimize preoperative planning and operative technique to maximizing oncologic and functional outcomes (Klatte et al., 2015).

The presence of inferior polar renal arteries might also be implicated as an etiologic factor in some forms of hydronephrosis correctable by surgery (Bergman et al., 2018).

These, as so many other examples, can be quoted to underline the importance of vascular anatomy in common and uncommon medical conditions, as well as its importance in surgical planning and outcome. Last but not least, we want to mention the importance of a universally accepted medical nomenclature in order to allow a faster and a more precise lecture and transmission of results in clinical practice, which will also facilitate the construction of research data bases based on objective anatomical descriptions.

Conclusions

Almost 20% of the population has multiple renal arteries, being double renal arteries the most common variation. Lateralization of the MRA was described in only a minority of the reported data, and no side's predilection was found among them. Only 21 articles described early branching patterns. It was described in 11,4% of the kidneys taken into account in those articles (4,23% corresponded to right and 4,52% to left kidneys. In 2,66% of the early branching cases there was no right/left specifications).

Due to the importance in clinical and surgical areas as different as nephrology, urology, oncological or vascular surgery, among others, the existence of these variations and its clinical implications should be taken into account by every physician.

To our knowledge, the present work is the widest anatomical review on the renal artery, with the most varied population.

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Circulatory system

The muscles of the athletes to learn surface anatomy - The Influence of classical statues on anatomy teaching

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Abstract

Gross anatomy classes are still regarded as an integral part of human biomedical education worldwide. The first documentary evidence of the practice of anatomical dissection for teaching purposes dates back to the 13th century AD, although this practice seems to have originated in Ancient Greece, if not in earlier times. Dissection of the human body is practiced in most anatomy institutions worldwide despite increasing pressure to reduce material and staff costs, regardless the ongoing debate concerning the suitability of body donors for medical education. Moreover, anatomical teaching skills are also evolving and need to be tailored for the different areas of anatomical expertise students have to acquire: therefore, anatomic dissection goes probably beyond the scope of anatomy teaching in some classes such as sports sciences. However, there is no doubt that a practical approach to the study and teaching of anatomy is surely preferable to basic *ex cathedra* anatomy lectures. Here, we propose a new teaching method for sports sciences and fine arts students by training their surface anatomy skills through the study of ancient statues.

Keywords

Anatomy teaching, dissection, muscular hypermorphism, surface anatomy, human anatomy education.

Introduction

Physicians and health practitioners have been inherently and historically captivated by human anatomy whenever they seek for answers that can help unravel our bodies's most hidden secrets.

This journey towards self-discovery generally begins systematically in medical school, where the study of human anatomy was once considered a cornerstone of medical education. The subject has been, to some degree, de-emphasized over the years in order to save time for other priorities in a fast-packed, modern curriculum

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(Drake, 2009; Meral Savran et al., 2015; McMenamin et al., 2016; Losco et al., 2017; Wilson et al., 2018.)

The ways in which medical educators teach the subject is also evolving: traditional anatomy education, based on topographical structural anatomy taught in lectures and gross dissection classes, has been replaced by a multiple range of study modules, including problem-based learning, plastic models or computer-assisted learning and curricula integration (Louw et al., 2009; Papa and Vaccarezza, 2013; Yammine, 2014; Chan and Pawlina, 2015; Moro et al., 2017; Periya, 2017).

In 1235 AD, when the first medical school opened in Salerno, Italy, anatomy rose to a prominent position in the medical curriculum and human dissection was performed as a sacramental procedure that illustrated the dissertations of revered ancient authors.

Recently, the idea that the Middle Ages were a time of obscurantism and decadence has been revised: the middle-to-late Middle Ages were indeed marked by many scientific accomplishments (Mavrodi and Paraskevas, 2014; Papa et al., 2019). The late 11th century and 12th century saw the establishment of a number of universities across Europe, including the University of Bologna.

Known as the ''Restorer of Anatomy'' (Crivellato and Ribatti, 2006; Rengachary et al., 2009), Mondino de' Liuzzi (1275-1326) is considered the first to have performed a dissection after Herophilus (c. 335-c. 280 BC) and Erasistratus (fl. 250 BC) were active in Alexandria (Ptolemaic Egypt). Dissection took place in 1315 on an executed criminal, most likely a woman, and was observed by medical students and the public with the purpose to indicate the exact position of the anatomical elements described by Galen. According to the custom of that time, Mondino did not perform the dissection himself. Because of his distinguished status, the professor sat on a large, elevated chair above the dissection table, reading aloud from Galen's book of anatomy and commenting commenting on it for the benefit of the audience. While he was reading, a barber-surgeon performed the dissection following the professor's instructions (Di Matteo et al., 2017; Mavrodi and Paraskevas, 2014).

When a cadaver was made available, time became a capital issue, since there were no effective means of preserving it. This is why the abdominal cavity, which contained organs that putrefied most easily, was dissected first, followed by thorax, head, and extremities.

Mondino's book "Anothomia" was completed a round 1316 and for at least two centuries it remained a classical anatomical textbook used by all major European universities (Crivellato E., Ribatti D. 2006).

During the Renaissance, after the opening of the Anatomical Theatres in Padua (1490) and Bologna (1637), anatomy was considered an artistic and spiritual exploration of life, suffering, and death. These theatres were multifunctional: a place to understand human anatomy, to witness the exaltation of life through the analysis of death and to be captivated by science.

Anatomists began to dissect in order to investigate the structure of the body and produced texts illustrated with images based on their dissections (Porter, 2002; Richardson, 2000; Hunter, 2001).

In the 16th century Vesalius changed the face of anatomical studies and teaching with his observational studies on dissected human tissues and the consequent publication of *De Humani Corporis Fabrica*. Vesalius's work put the exquisite detail

and three-dimensional form of the human body onto paper. His work and publications captivated, engaged and educated scholars and students and set the standard for subsequent generations of anatomical publications, research and training (Kemp, 2010; Riva et al., 2010; Eisma et al., 2013).

The text and iconography of Vesalius' *Fabrica* had a tremendous influence on medical thinking since its publication in 1543. The reasons were manifold: the visualization of natural and realistic human anatomy rather than anatomy that was theologically-inspired, the magnificent Renaissance depiction of the human body in different poses and in various stages of dissection, the unprecedented use of anatomical terminology, the classification into seven organ systems, and the reaction against the millennium-old theories of Galen (Van Hee et al., 2014).

One of the greatest challenges in anatomical studies is gaining appreciation of the three-dimensional nature of anatomical structures and their positional relationships. Achieving this from books and two-dimensional imaging is difficult and is, therefore, supplemented with the study of cadaveric specimen or plastic models. In the 17th century, as anatomical studies became more scientifically based, the first attempts to use injection to preserve anatomical preparations of the human body from deterioration were carried out by the Italian physician Marcello Malpighi (1628–1694). The Dutch naturalist Jan Swammerdam (1632–1680), too, used both colourless or coloured preserving chemical fluids composed of alcohol, mercury, different metals (including lead, tin, bismuth) and wax. The results of these trials were reasonable, although preparations were not durable and subsequently deteriorated.

Towards the end of the 17th century a collaboration between Gaetano Giulio Zumbo (1656-1701), a Sicilian wax artist, and the French surgeon Guillaume Desnoues (ca. 1650-1735) resulted in the creation of the first realistic anatomical models made from coloured wax, representing a valid alternative to dissected human specimens. Wax gradually took its place as a material capable of allowing the creation of extremely realistic (as to form and colour) and long-lasting artefacts (Ballestriero, 2010).

In 1977 von Hagens et al. introduced plastination, a new technique of tissue preservation: in this process water and lipids in biological tissues are replaced by polymers able to determine the mechanical and optical properties of the specimens. The resulting material is odorless and durable and retains structural and morphological details down to the histological level.

Lastly, a new method of embalming bodies named Thiel method is worth mentioning (Thiel, 1992). This technique, developed by Walter Thiel at the University of Graz (Austria), is still not widely known and needs careful procedures and dedicated structures (Benkhadra et al., 2011; Eisma et al., 2011, 2013), but it looks promising to study the skeletal and the muscular systems in a more physiological and dynamic way and it is reported to be one of the best training tools in the field of anatomical surgery (Hammer et al., 2015).

Unfortunately, muscle protein fragmentation is relevant and thought to cause the flexibility of the Thiel embalmed bodies. Therefore, Thiel fixed specimens are not suitable for histological analysis nor biomechanical testing (Fessel et al., 2011; Hammer et al., 2014).

Mazzotti et al. (2010) clearly demonstrated the proper use of an anatomical model for teaching and diagnostic purposes: they studied *Venerina*, a wax removable model

by Clemente Susini representing an accurate copy of the original anatomical preparation of a pregnant young woman. The model is about 145 cm tall and the pelvis shows an opened pregnant uterus with fetus and placenta; the fetus is about 15 cm crown-rump length, from which it has been estimated that the woman was in the fifth month of her pregnancy.

Observation of the body confirms that the organs are normal, except for the heart and great vessels: the walls of both ventricles are of equal thickness and the ventricles themselves of approximately equal size. The arch of the aorta and the enlarged pulmonary trunk are connected by a short duct about 3.5 mm in diameter originated at the level of the division of the pulmonary artery, reaching the aortic arch at the beginning of the descending part and corresponding to the normal insertion of the arterial duct that, in the fetus, connects the two great vessels; after birth it closes and becomes the arterial ligament. If the young woman really died from this congenital disease, her cause of death was surprisingly diagnosed from a wax model of her body after more than two centuries.

Furthermore, Galassi et al. (2015), examining the whole Bologna collection, stressed the importance of anatomical waxes and suggested a reintroduction of these models in the medical curriculum.

The effectiveness of arts-based approaches in medical education has been discussed for over 50 years. The assumption underlying the use of the arts in medical education appears to be that the arts can assist in the development of the student as a communicative doctor through two main theoretical mechanisms. Firstly, study of the arts can directly provide students with a 'simulation' of the wider experience of life necessary for mature interaction with other human beings, which might otherwise be unavailable to them. Secondly, direct participation in the artistic process may help students to explore their own feelings, question them, and develop new ways of thinking. Exposure to the arts has also been used to improve technical skills such as diagnostic observation skills (Perry et al., 2011).

Surface anatomy is an essential component of the study of the human body (Leonard, 1996; Standring, 2012). It is not just about knowing what lies under the skin and which structures are perceptible to touch in a living body, it is also about enabling learners to improve their skills in clinical examinations, interventional procedures, and interpretation of diagnosing images. Studying surface anatomy enables students to elaborate on their knowledge of the cadaver's static anatomy by enabling the visualization of how structures, especially those of the musculoskeletal system, move and function in a living human being (Barrows et al., 1968; Bergman et al., 2013).

Surface anatomy was not introduced into anatomy education until well into the 1940s (McLachlan and Patten, 2006). The first studies of the use of live models in anatomy education were published more than 40 years later (Barrows et al., 1968; Stillman et al., 1978). Nowadays, surface anatomy is promoted as one of the most important modalities contributing to an optimal learning content of anatomy education (Sugand et al., 2010). Studying surface anatomy provides an elaboration on the static anatomy of the cadaver by enabling students to see phenotypical structures, particularly those of the musculoskeletal system, move and function in a living human being. Furthermore, surface anatomy sessions enable students to familiarize themselves with important surface landmarks and to observe, examine, and interact

with a living person. In some institutions, surface anatomy is therefore thought to be of vital importance for education, sometimes even more important than teaching anatomy with cadavers (Monkhouse, 1992).

A recent development within surface anatomy education is body painting. The concept of painting on a live model was described by Cody (1995), but Op Den Akker et al. (2002) were the first to introduce body painting as a teaching tool. In body painting sessions, hypo-allergenic, water-based body paint is used by students to paint anatomical structures and/or concepts (e.g. dermatomes) on their peers under supervision by anatomy staff (McMenamin, 2008; Azer, 2013).

However, to the best of our knowledge, no studies other than body painting papers concerning the arts-based approach to train surface anatomy have been published nowadays.

The use of wax models and reproduction of human bodies (such as sculptures) could help teaching anatomy according to the different skills students have to acquire. It is also worth mentioning that the teaching of anatomy should be arranged according to the different skills students need to use in their future practice: anatomical dissection is therefore beyond the scope of anatomy teaching in some classes such as sports sciences.

According to Lambertini (1936): "*often missing in the teaching of anatomy is the in vivo topography of the muscle so that the student does not appreciate the value of the muscle in a dynamic and aesthetic way and therefore s/he is unable to catch its functional value properly*". Moreover, "*the main topic in fine art is the human figure represented by the means of plans, lines and color and therefore body structure, bones and joints, muscles and tendons and all that is by nature in motion reduced to the stiffness of the drawing* " (Della Seta, 1930).

Keeping these assumptions in mind, it could be a smart move to practice surface anatomy on wax reproductions and sculptures: studying statues' surface anatomy overcomes anatomy of the cadaver by enabling students to analyse musculoskeletal system structures; moreover surface anatomy sessions enable students to familiarize themselves with important surface landmarks and the statue itself can be considered as a 3D moving model of a human being.

Materials and Methods

In July 2013, an important ancient statue from the National Archaeological Museum of Naples (MANN) moved to Cassino during the National University Championships, supported and sponsored by University of Cassino and Southern Lazio.

The statue itself is a sculpture of unknown identity, found in 1936 in the Roman theatre of Cassino (Lazio) and became part of the National Museum of Naples collection (Zanker, 2010).

It was originally known as "General of Cassino" was later renamed "Athlete of Cassino" because of its defined athletic *physique*.

The statue dates back to the mid first century BC and portrays a mature man, clean-shaved, with a mane of short hair adhering to the head and accentuated receding hairline; wrinkles on the forehead and eyebrows raised help to provide the impression of concentration and awareness (see figs. 1-3). Quite in contrast to the head and the face, the naked body appears in resting position with one leg raised

Table 1. Length of statue body segments (cm). Statue scale 1:120 (human male 1,75-1,80 m tall)

Styloid process-Olecranon of the ulna	37 cm
Olecranon-Acromion	50 cm
Trochanter-Meniscus	61 cm
Meniscus-Lateral Malleolus	49 cm

with the foot on a rock, the arms crossed and a cloak to cover what remains of the hilt of a sword, ready to be drawn.

The representation into sharp relief of the *serratus magnus* muscle, typical of classical sculpture, requires a deep knowledge of human anatomy, giving support to the hypothesis that dissection was practiced by classical sculptors, possibilities generally denied or ignored by scholars due to repulsion and prejudice rather than to the lack of sources.

The sculpture belongs to the type of statues depicting an athletic body combined with a portrait representing the real face of the purchaser and thus reflecting the aesthetic concept which aimed to give heroic aura to eminent Romans through athletic nudity.

We cannot interpret this statue simply as the physical embodiment of personal inspiration to power but it implies inspiration from the Hellenic model and becomes far more impressive than that of traditional togate statues (Zanker, 2010). These properties make this sculpture apt for analysis of the use the statue dynamics and its 3D posture to teach and train surface anatomy.

Anatomy courses are often delivered to sport sciences undergraduates as basic classes during the first and the second year. Unfortunately, in undergraduate courses such as sports sciences (especially in non-English speaking countries such as Italy) practical sessions are less and less implemented, meaning that presentation notes, textbooks, atlas or 3D devices are the only learning tools available for students.

In this reassessment, we therefore propose to teach surface anatomy to sport and fine art students through the evaluation of ancient statues (where available).

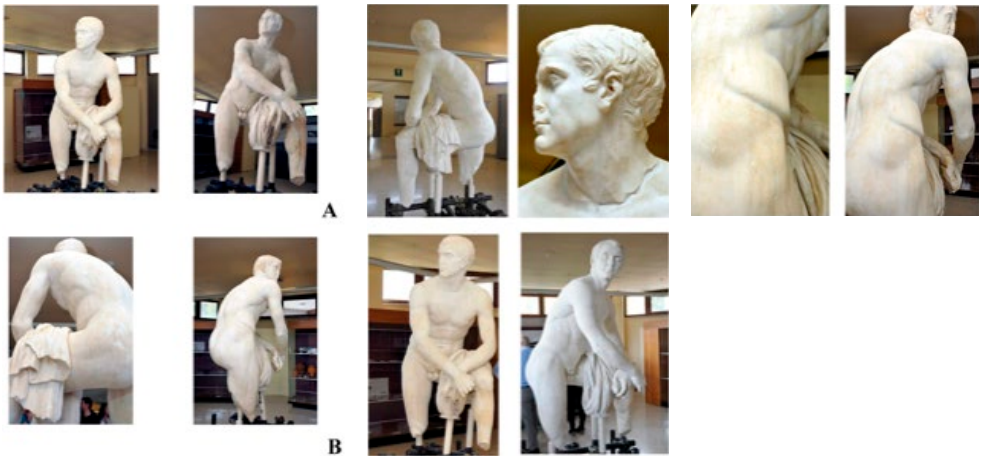
The preliminary assessment of the statue was performed by both sport sciences and art and literature undergraduate students of the University of Cassino and Southern Lazio under the supervision of some of the authors (VP; AR).

Anthropometric features were measured using a flat tape in order to study surface anatomy (Asad and Nasir, 2015). The results are shown in Table 1.

Results and Discussion

As shown in figure 1, the athlete does not present hypertrophy of the deltoid, trapezius, biceps and triceps muscles, typical features of throwing sports, while it seems that lumbar sacral muscles are symmetrically developed, which is a specific feature of dexterity, agility and combat sports athletes (Holmberg, 2009; Zemková, 2014).

According to Lambertini (Lambertini, 1936) the muscular hypertrophy of specific muscle fascicles caused by an overactive, continuous and peculiar exercise of specific muscle groups in certain sports can be defined as muscle hyper-morphisms. Muscle



Figures 1-3. Unknown artist, "the Athlete of Cassino", 1st century BC, Naple's National Archaeological Museum (MANN), Naples, Italy; G. Carettoni Museum, Cassino, Italy. Photo credits: D. Marino, E. Polito. By concession of the Ministry of Cultural Goods and Activities and of Tourism - National Archaeological Museum of Naples.

hyper-morphisms therefore testify the practice of a particular and distinctive sport practice.

The idea that the practice of different sports could result in a preferential, not exclusive and characteristic muscle groups activation was indeed already deeply and firmly established in the 5th Century BC: in Polykleitos' Doryphoros, the *pectoralis major*, *serratus anterior*, *rectus abdominis* and the obliquus externus abdominis muscles show, in fact, the hyper-morphisms of momentum sports.

The here discussed athlete does not present, compared to others such as Polykleitos' Doryphoros, a slender physical appearance but a squat, short-limbed phenotype similar to Agasias' Fighting warrior. [Louvre Museum official site: http://cartelen.louvre.fr/cartelen/visite?srv=car_not_frame&idNotice=17178, accessed February 20, 2018.]

The Athlete of Cassino is in fact characterized by symmetrical appearance of hypertrophic muscles capable of strong, stocky and long lasting contractions even if muscles themselves are poorly sculpted and defined in shape. Moreover, a fair amount of adipose tissue remains under the skin as the morphology of the *rectus abdominis* seems to demonstrate.

Furthermore, according to the hyper-morphisms characterizing combat and fighting sports, he presents harmonic development and right proportions between the upper and lower part of the body.

The spine and back features of combat athletes typically include a significant increase in volume of all of the muscles responsible for extension of the spine (*latissimus dorsi* and the intrinsic spinal muscles) as well as the *gluteus maximus*, thigh hip extensor.

As far as we are concerned about what is a general feature of a wrestler, the statue, due to its posture, does not present hypertrophy of the trapezius. In combat ath-

Figure 4-5. Polykleitos, "Doryphoros", II-I century BC., MANN, Naples, Italy. Photo credits: A. Panarello, V. Papa. By concession of the Ministry of Cultural Goods and Activities and of Tourism - National Archaeological Museum of Naples.

letes, in fact, the *trapezius* typically thickens widening its belly at the spinous processes of the thoracic vertebrae, origin of its transverse portion (figs. 1B and 2).

As would be expected, the full musculature of both anterior thorax and the abdominal region shows an impressive gain in volume and shape. Especially in contact sports, the thorax and abdomen muscles involved in respiration have to increase their activity because of the multiple "*bear hugs*", a rough, tight embrace typical of wrestling moves occurring in wrestling fights.

As also shown in fig 2, in fact, *sternocleidomastoid* and *infrahyoid muscles* typically gain in shape and volume since the wrestlers and the boxers look after their face and head, therefore covering themselves with their arms and flexing the head.

The clavicular head of the left *sternocleidomastoid* can be accurately identified whilst the right one is hidden between the skinfolds of the neck; at the same time both the upper edge of the left clavicle up to its acromial end and the jugular notch of the sternum, which is well designed in its three portions, are clearly visible. The epigastric arch is not clearly delineated while bordering with *latissimus dorsi*, *serratus magnus* is undoubtedly well defined (fig. 1).

Furthermore, deep longitudinal grooves, the *linea semilunaris* or *Spigelian line* are clearly visible lateral to *linea alba* separating the *rectum* from oblique muscles; the latter, on the right side, is particularly defined in shape and almost seems to protrude from the iliac crest (fig. 3) being in close continuity with the *gluteus maximus* and *tensor fasciae latae (*fig. 3).

Lastly a brief mention of the Polykleito's Doryphoros needs to be made, mostly in order to better understand the hyper-morphisms differences between the two artworks.

We analyzed the Doryphoros housed in the National Museum of Naples which is one of the best among the many ancient replicas (Della Seta, 1930).

Sporting activities such as discus, hammer or javelin throw, determine asymmetric hyper-morphisms which do not comply with bilateral symmetry: the muscles of the dominant body part are therefore more defined in shape than those in the non-dominant side.

The statue distributes its body weight on the right leg according to a pattern which is thought to have been introduced by Polykleitos himself; the left leg is slightly slid and carried back, the support is provided only by the front of the foot. The athlete holds the javelin (this being a modern recreation of the statue) into the left hand while the right leg distributing the body weight slightly leaps forward according to the idea the athlete was left handed and maybe getting ready for his skill (fig. 4).

Furthermore, the *serratus magnus* and the external oblique muscle of the abdomen which partially originates on the ribs are more defined in shape on the left side rather than on the right one; the latter protruding out of the iliac border. The *pectoralis major, serratus magnus, external oblique* and *rectus abdominis* muscles are also represented according to Lambertini's hyper-morphisms definition of asymmetric sports (fig. 5) (Della Seta 1930; Lambertini, 1936; Moon, 1995).

Conclusion

Although we and others have previously suggested that dissection and prosection need to remain essential tools in anatomy teaching even in modern medical curricula and clinical training, it is also undoubtedly true that it is necessary to examine the curriculum, the mode of teaching, the quality of how it is delivered, and the infrastructure within which it is delivered for optimal and proficient tailoring of anatomy teaching and learning material. Considering the most recent trends in anatomy pedagogy where at least in a short term learning all the various anatomy learning tools seem to be equivalent (including dissection, Losco et al., 2017,; Wilson et al., 2018), we therefore strongly suggest that surface anatomy training on models such as ancient statues could be considered a valuable tool along with surface anatomy training in those classes in which dissection is independent from the purpose of the study course as sports sciences and fine arts. This additional tool, related to statue and models availability, should become a relevant part of the anatomy "armamentarium" for learning and teaching.

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affiliation, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Nervous system

Topographical and surgical anatomy of third cranial nerve. A review

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Abstract

Knowledge of the neuroanatomy of the third cranial nerve, oculomotor nerve, which provides motor innervation to four of the six extraocular muscles and the levator palpebrae superioris, and parasympathetic innervation to the sphincter pupillae and ciliary muscles, is critical for neurosurgical management of lesions located in the cavernous sinus or orbit. The oculomotor nerve itself has a complex anatomy regarding anatomical landmarks for its localization, considering the characteristics of the arachnoidal sleeve and cisterns that accompany the oculomotor nerve especially through the cavernous sinus. The aim of this review was to underline anatomical landmarks for localization of the ocumolotor nerve.

Keywords

Oculomotor nerve, neuroanatomy, surgical anatomy, neurosurgery.

Introduction

Knowledge of the neuroanatomy of the III cranial nerve, oculomotor nerve, which provides motor innervation to four of the six extraocular muscles and the levator palpebrae superioris, and parasympathetic innervation to the sphincter pupillae and ciliary muscles, is critical for neurosurgical management of lesions located in the cavernous sinus or orbit.

The aim of this review is to underline anatomical landmarks for localization of the ocumolotor nerve, considering the characteristics of the arachnoidal sleeve and cisterns that accompany the oculomotor nerve especially through the cavernous sinus.

Material and methods

A systematic literature review of English articles since 1965 was performed using search terms pertaining to oculomotor nerve topography, surgical triangles, cisterns and cavernous sinus anatomy.

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Results

The nuclei of origin of cranial nerve III, the triangular-shaped oculomotor nuclear complex, is located in the mesencephalon. Edinger (Edinger, 1885) and Westphal (Westphal, 1887) described the small-cell nuclei which still commemorate them, and in 1889 Perlia (Perlia, 1889) combined these and his own topographic findings to produce his well-known diagram of the oculomotor nucleous. He added another paired, small-cell group, the anteromedian nucleus, situated cranially and in the midline, and caudal to this his better known "central" nucleus. The oculomotor nucleus consists of an elongated mass of cells lying ventral to the periaqueductal gray matter adjoins the trochlear nucleus rostrally at the ventral border of the periaqueductal gray matter and extends rostrally to the level of the posterior commissure (Büttner-Ennever, 2006).

Unlike the troclear and abducens nuclei, the oculomotor nucleus has both midline unpaired and lateral paired portions. It consists of several cell groups: the symmetrically paired lateral nuclei, which provide the innervation of the extrinsic ocular muscles, and the symmetrically paired parasympathetic nuclei, situated above, medial to and in front of the preceding nuclei, which provide the innervation of the intrinsic ocular muscles.

Fascicles of the oculomotor nerve originate from the entire rostral-caudal extent of the nucleus and pass ventrally through the medial longitudinal fasciculus, red nucleus, substantia nigra, and medial part of the cerebral peduncle. As they pass through the red nucleus, the fascicles fan out, and converge again before exiting the midbrain in the interpeduncular fossa. Based on clinicoradiologic and clinicopathological findings, it is proposed that, from lateral to medial, the order of fascicles is inferior oblique, superior rectus, medial rectus and levator palpebrae, inferior rectus, and pupil (Gauntt et al., 1995).

The apparent origin of the oculomotor nerve is from the anterior aspect of the brainstem, at the medial border of the base of the cerebral peduncle. After leaving the cerebral peduncle and the posterior perforate substance the nerve courses between the posterior cerebral artery and superior cerebellar artery and passes forward, downward, and laterally via the basal cistern (Adler and Milhorat, 2002).

The nerve passes inferolateral to the posterior communicating artery, above the superior cerebellar artery, below the temporal lobe uncus, where it runs over the petroclinoid ligament, medial to the trochlear nerve and just lateral to the posterior clinoid process. An arachnoidal reinforcement is there where the posterior communicating artery penetrates the interpenducular cistern and the oculomotor nerve with its own arachnoid sheath, leaves the cistern to enter the dura of the cavernous sinus. Dense arachnoid trabeculae often bind the artery and nerve to each other at this point (Yasargil et al., 1984). The nerve glides under the posterior clinoid process, producing a more or less marked sulcus on the lateral margin of the dorsum sellae, and then enters the transverse plate of the cavernous sinus at the "pore" for the oculomotor nerve. The anterolateral margin of the oculomotor pore is sharp. A pocket of dura and arachnoid accompanies the nerve for a distance of 6-8 mm into the cavernous sinus (Lang, 1983). During the subarachnoid course, parasympathetic pupillary fibers lay peripherally in the dorsomedial part of the nerve (Sunderland and Hughes, 1946).

Within the wall of the cavernous sinus, the third cranial nerve lies initially above the trochlear nerve, where it receives sympathetic fibers from the carotid artery. It

does not become incorporated into the fibrous dural cavernous sinus wall until it reaches the lower margin of the anterior clinoid process. The lower cavernous sinus is an interperiosteo-dural space limited laterally by a meningeal layer. The medial wall is composed of 2 parts: the sphenoidal part, which corresponds to the endosteal layer, and the hypophyseal part, which corresponds to the encephalic (meningeal) layer. (François et al., 2010).

After leaving the cavernous sinus, the oculomotor nerve is crossed superiorly by the trochlear and abducens nerves and divides into superior and inferior rami, which pass through the superior orbital fissure (Natori and Rhoton, 1995), and enter the orbit within the annulus of Zinn. The superior oculomotor division runs lateral to the optic nerve and ophthalmic artery, to supply the superior rectus and levator palpebrae muscles from their global sides. The larger inferior oculomotor division branches in the posterior orbit, to supply the medial rectus, inferior rectus and inferior oblique muscles and the ciliary ganglion (Sacks, 1983).

Crucial to the surgeons understanding of the relevant surgical anatomy of the cavernous sinus is a working knowledge of the multiple triangular shaped entry corridors into the region (Fukushima, 2011).

The oculomotor nerve delineates three of the cavernous sinus surgical triangles: clinoidal (anteromedial), oculomotor (medial or Hakuba's) and supratrochlear (paramedian or Fukushima's) triangles

The clinoidal triangle is bounded by the optic neve, oculomotor nerve before entering the superior orbital fissure and from the dural fold between dural entries of optic and oculomotor nerves. The clinoidal triangle is visible after removing the anterior clinoid process. It is limited laterally by the oculomotor nerve and medially by the lateral margin of the optic nerve. It is in close relationships with the clinoid segment of the internal carotid artery.

The oculomotor triangle is where the oculomotor nerve enters the roof of the cavernous sinus. The corners of this space are the anterior and posterior clinoid processes and the petrous apex. These points are connected by the anterior and posterior petroclinoid folds and the interclinoid dural fold. The space bounded by this triangle exposes the distal intracavernous carotid artery and is an important access corridor for tumors involving the medial cavernous sinus and for approaches to the interpeduncular fossa.

The supratroclear triangle is bounded by the III and IV cranial nerves with its posterior margin being the crest of dura at the transition from medial to posterior fossa. This triangle is the most suitable for exposure of the carotid cavernous segment-clinoid segment junction (Fukushima, 2011). The supratroclear triangle is defined by the oculomotor (located above) and trochlear (located bellow) nerves, it is called medial triangle by some authors and has the following corners: subclinoidal carotid segment, posterior clinoid process, and the oculomotor porus.

The oculomotor cistern courses from the oculomotor porus to the inferior surface of the anterior clinoid process. The segment of the nerve inside the oculomotor cistern is interposed between its free portion in the interpeduncular cistern and the part of its course where it is incorporated into the fibrous lateral wall of the cavernous sinus. This small dura-arachnoid cuff is filled with cerebrospinal fluid and is an important surgical and imaging landmark. The nerve in its proximal intracisternal portion averages a diameter 2.5-3.0 mm, is round. Progressively the nerve towards and down-

wards the cavernous sinus becomes flattened. Its intracisternal cross section area is on the right 2.90 - 4.55 mm^2 and on the left 2.90 - 4.22 mm^2 (Thorsteinsdottir, 1982).

In its cisternal course after emergence from the brain stem the oculomotor nerve receives arterial supply from branches of the vertebrobasilar system, may receive arterial supply in the vicinity of the posterior perforating substance from the basilar artery or from the posterior cerebral artery. The blood supply of the intracranial portion of the oculomotor nerve from its emergence from the brainstem until it passes over the posterior cerebral artery originates from thalamic perforating branches. The middle intracranial part of the nerve does not receive nutrient arterioles from adiacent arteries (Fukushima, 2011).

Dural arteries contribute to the supply of the nerve in its distal dural and transosseous course. On the roof of the cavernous sinus blood is supplied by the marginal artery of the cerebellar tentorium which usually is a branch of the meningohypophyseal trunk. The marginal artery of the tentorium is an arcade between orbital vessels and branches of the carotid siphon. Many anatomic variations may be observed in this region (Lasjaunias, 2001).

Discussion

Cranial nerve III injury most likely occurs during cavernous sinus surgery or during surgery in the region of the interpeduncular fossa. Injury is one of the leading postoperative ophthalmological morbidities following cavernous sinus surgery and may occur during some of the steps in approaching benign cavernous sinus tumors after splitting the Sylvian fissure: (a) resection of the dura and underling anterior clinoid and lateral cavernous sinus wall; (b) resection of the optic strut and exposure of the orbital apex; (c) piecemeal resection of the tumor to expose cranial nerve V and its branches, cranial nerve III and cranial nerve IV (Sekhar et al., 1987). Due to its short intracavernous course, its anatomical continuity can often be preserved. However, it is very fragile and temporary functional impairment can occur with minimal manipulation. Success of third nerve regeneration after operative repair appears to be limited due to its highly differentiated functions. Aberrant regeneration of the oculomotor nerve following surgical intervention is likely more common than current literature would suggest (Weber and Newman, 2007).

The evolution of technology promote new studies as the use of two and three dimensional educational materials for accurate study of the triangles and nearby structures trying to analyse further the surgical anatomy of the cavernous sinus (Beom Sun Chung et al., 2016)

All these points support the usefulness of continuous anatomical knowledge improvement through theoretical and practical preparation for neurosurgeons to obtain the best preservation of oculomotor nerve.

Conclusion

The oculomotor nerve itself has a complex anatomy regarding anatomical landmarks for localization of the nerve, considering the characteristics of the arachnoi-

dal sleeve and cisterns that accompany it especially through the cavernous sinus. This makes consistent landmarks difficult to identify, therefore it is necessary that neurosurgeons have the most complete knowledge of the surgical and functional anatomy of this nerve to obtain the best preservation during neurosurgical operations.

Advanced section images that allow identification of almost all structures in the entire courses of III, IV, and VI cranial nerves from the central to the peripheral nervous system can inform on the potential ability of such images for the construction of sophisticated three-dimensional models of those nerves from the brainstem to the orbit (Park et al., 2015). Technologically advanced cadaveric dissections supported by high resolution images (7 Tesla magnetic resonance imaging) and appropriate neurosurgical procedures could improve the already available knowledge regarding the surgical anatomy of the III cranial nerve (Fukushima, 2011).

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Circulatory system

Relationships between seasonal (spring, summer, autumnal) thermal variations and cell proliferation in heterothermic vertebrates, as revealed by PCNA expression in the brain of adult *Podarcis sicula*

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Abstract

Among the literature reports on the possible effects of the seasonal cycle, made of temperature and photoperiod variations, on spontaneous proliferation in the brain of adult fresh water, earth-dwelling Anamnia and heterothermic Amniota, one autoradiographic study was conducted on experimentally brain injured and normal *Rana esculenta*, collected in the wild in spring and in autumn, and another immunohistochemical study was conducted on brain injured *Podarcis hispanica* caught in nature in summer. To expand that knowledge an immunohistochemical investigation has been performed on brain of normal adult *Podarcis sicula* captured in the wild in spring, summer and autumn with the aim to analyse exclusively the seasonal (temperature and photoperiodic) cycle impact on latent spontaneous proliferation. Cycling cells have been labeled for PCNA. The results show that the cycling cells are rare in spring, in intermediate numbers in summer and frequent in autumn. Therefore environmental conditions affect the proliferative capacity of the cells in stand-by, that are typically mainly positioned in some telencephalic areas: the *zonae germinativae latero-dorsales*, *medio-dorsales* and *ventrales*. An investigation on the winter aspect was purposely omitted, since such lack of cycling cells in winter was already known from previous literature reports. With the present findings the time course of proliferation of putative brain stem cells - as demonstrated by immunolabeling for PCNA - is assessed in lizard for the whole year.

Keywords

Season influence, neural matrix cells/areas, *Podarcis*.

Introduction

The plasticity of adult brain has been ascertained for some anamniotic and amniotic vertebrates, mainly in fresh water fish, like Teleosts, in earth-dwelling Amphibia, like Urodeles and Anurans, and in terrestrial Reptiles, like lacertilians. That plasticity depends on the presence of surviving stem cells, which are responsible for the persistence of proliferative potential and therefore, probably, of reparative and even regenerative power.

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The proliferative potential is linked to the presence of small, basophilic, neural-like cells, that are remnants of the germinative neural layer of early embryonic development (Kahle, 1951; Fujita, 1963; Kirsche, 1967). Their number decreases from earlier to more advanced embryonic stages, then to the subsequent larval stages, if present, and eventually to adult life.

The number of these sleeping cells can vary among the different vertebrates; generally speaking they appear much grater in lower vertebrates than in higher ones. An increasing plasticity is shown from lacertilian Reptiles to anuran Amphibians to Teleosts to urodelan Amphibians; the last animals are the vertebrates most gifted with putative stem cells.

These putative precursor or stem cell are normally silent, but they are capable of self-reproduction and can start cycling again giving rise to descendants which may undergo late differentiation into neuronal or glial cells (Kirsche, 1967, 1983).

An exhaustive, comparative, autoradiographic study was carried out by Kirsche (1967) on the normal adult brain of vertebrates, ranging from Teleosts to Birds passing through urodelan and anuran Amphibians and lacertilian Reptiles and focusing on the presence of such cells in stand-by which can be found scattered ("matrix cells") or clustered ("matrix areas") in the layers lining the cerebral cavities. In details, the matrix areas ("Matrixzonen" according to Kirsche, 1967) are at the dorsal and ventral edges and at the bottom of each ventricular surface of the telencephalic hemispheres, making up, respectively, the *zonae germinativae dorsales* and *ventrales*, extended antero-posteriorly. The latter areas are generally wider and more populated in cells, whose persistence also appears more prolonged than those in the other areas (Kirsche, 1967).

In the meantime, Kirsche (1967) testified proliferative events in the *medulla oblongata* of *Triturus cristatus carnifex*, *T. vulgaris* and *Rana esculenta*, linked to the presence of matrix cells.

Subsequently, the presence of stem cells was reported in other encephalic sites and in other animal species mainly by autoradiographic, rarely by immunohistochemistry. In the layers delimiting the cerebral ventricles of Petromyzontidae (Margotta et al., 2007) and Selacians (Margotta, 2007), in ependymal and sub-ependymal layers of the olfactory districts of Teleosts, urodelan and anuran Amphibians and lacertilians Reptiles (Alonso et al., 1989; Garcia-Verdugo et al., 1989, Byrd and Brunjes, 2001; Margotta et al., 2005). In lacertilian Reptiles each *zona germinativa dorsalis* is distinguishable into two portions, *lateralis* and *medialis* (Minelli and Del Grande, 1980). A midbrain additional symmetrical matrix area (*zona germinativa caudalis*) was described in Teleosts, as well as matrix cells in the deep cerebellar tissues of Teleosts. Grouped matrix cells were found scattered in the forebrain of male songbirds ("hot spots") and "matrix tissue" was described in some Mammals (for further literature review see Margotta and Morelli, 1996).

Most investigations included submitting the samples to surgical ablations of encephalic plugs or areas, some were based on heterotopic hetero- (rarely homo-) transplants, *in vitro* culture of cerebral tissues and other experimental conditions and only a few were made on normal specimens. Methods were at first traditional histology, then autoradiography, seldom electron microscopy or immunohistochemistry, the last method being applied to target proliferation-related enzyme activity.

The awareness on the plasticity of adult brain in fresh water and earth-dwelling Anamnia and poikilothermal Amniota was sometimes obtained evaluating, among

other issues, if a seasonal cycle, made of temperature and photoperiod variations, alone or coupled with various experimental procedures might activate proliferative fluctuations or unmask an encephalic latent spontaneous proliferative potential, thus showing reparative and even regenerative potentiality due to an otherwise hidden mitotic activity of quiescent cells still present in the adult brain.

In particular, Minelli et al. (1982) in an autographic study on acutely injured and uninjured brain of adult *R. esculenta*, ascertained that the trend of labeled nucleside uptake both in brain-injured and normal specimens was extremely low in advanced spring, was higher in autumn and waned again in proximity of winter. This trend was inverted in spring and autumn by first submitting the samples to cold.

Ramirez et al. (1997), by autoradiography and immunostaining, made similar investigations on adult brain-injured *Podarcis hispanica* caught in nature in summer and stated that cerebral proliferation was increased in summer and migration of newly generated immature neurons was inhibited in winter.

These studies were incomplete in that one lacked information on summer events (Minelli et al., 1982) and the other lacked information on spring and autumn ones (Ramirez et al., 1997). Therefore we have now carried out a study on normal adult brain of *P. sicula* (once *Lacerta viridis* Rafinesque: Tortonese and Lanza, 1968), captured in nature in spring, summer and autumn. The study was also aimed at ascertaining if the findings of Minelli et al. (1982) for spring and autumn might be extended to adult *P. sicula*, *i.e.* across different species.

The present study was performed with an immunocytochemical method, by revealing the Proliferating Cell Nuclear Antigen (PCNA: Miyachi et al., 1978). This marker had previously proved to reliable and suitable as a proliferation test (for further details see Margotta and Chimenti, 2016).

Materials and methods

Normal adult *Podarcis sicula* - as ascertained according to Capula (2000) – have been involved in the actual research. On the whole, six samples (three males and three females) were considered for each season. All specimens were collected in the wild near Roma, Italy. The individuals here employed as controls belonged to past catches and related investigations: some were caught in late spring (environmental temperature between 10° and 16°C) (Margotta et al., 1999, 2005), others at the end of July (environmental temperature between 12° and 24°C) (Margotta, 2014). Furthermore, a portion of the specimens belonging to lizards of the second capture were maintained in a stable. put in an open environment, to prevent difficult availability (diapause) during advanced autumn, and then were sacrificed in that season (temperature between 8° and 18°C). The lizards were sacrificed under anaesthesia with tricaine methanesulfonate (Ms 222 Sandoz, Switzerland, 1:1000). The head was cut off and after partial disarticulation of the cranial bones it was fixed in Bouin's fluid and then transferred to 80% ethyl alcohol, where the brain was removed under a stereomicroscope. The tissue was dehydrated through graded ethyl alcohols, cleared in histolemon and embedded in paraffin under *vacuum*. Transverse, 8 μm thick serial sections were cut in antero-posterior direction with a rotary microtome.

For immunohistochemistry the sections were deparaffined and hydrated, rinsed in isotonic, 0.01 mol/litre phosphate buffered saline, pH 7.4 (PBS), incubated in 3% H_2O_2 in methanol for 30 min to block endogenous peroxidase, washed in PBS, incubated in 20% normal horse serum to block unspecific binding sites and incubated overnight at 4 °C in a monoclonal antibody against PCNA (PC10 mouse IgG, from Sigma, St. Louis, Missouri), diluted 1:1000 with PBS plus 1% normal horse serum. Negative control sections were incubated with non immune mouse IgG instead of the primary monoclonal. The bound antibodies were detected using secondary horse anti-mouse biotinylated antibodies (Vector, Burlingame, California), diluted 1:100 with PBS plus 1% normal horse serum, for 1 h at room temperature, and avidin-biotin-peroxidase complex (ABC Kit, Vector), 30 min at room temperature. Peroxidase was detected with 3-3'-diaminobenzidine tetrahydrochloride (DAB. Sigma) 1 mg/ml, plus 1% $NiSO_4$ and 0.017% H_2O_2 in 0.05 mol/litre Tris-HCl, pH 7.6. Slides were then dehydrated and mounted with Entellan (Merck, Germany).

Results

The actual account originate from an analysis carried out in normal adult specimens of *P. sicula*, in part from past catches in spring and at the end of July, previously published (Margotta et al., 1999, 2005, Margotta, 2014), and in part from present samples maintained in a stable kept in an open environment till advanced autumn before sacrifice. The following results were drawn.

In the olfactory peduncles PCNA positive cells appeared scanty in spring (Fig. 1a), numerous in summer (Fig. 1b) and even more abundant in autumn (Fig. 1c). These cells were scattered among the ependymal epithelium lining the ventricles, rarely were seen also in the sub-ependymal layer.

In each telencephalic hemisphere PCNA positive cells were found in areas located dorsally (each subdivided in two portions, lateral and medial) and ventrally, corresponding to *zonae germinativae latero-dorsales*, *medio-dorsales* (Figs. 2a, b, c) and *ventrales* (Figs. 3a, b, c) as known after Kirsche (1967), Minelli and Del Grande (1980). Few labeled cells were observed in spring (Figs. 2a, 3a), an intermediate number in summer (Figs, 2b, 3b) and many in autumn (Figs. 2c, 3c). The ventral matrix areas, in contrast to the dorsal ones, were more extended in antero-posterior direction and were better provided with putative stem cells, as anticipated by Kirsche (1967).

In the diencephalon of samples sacrificed in spring, summer and autumn weak labeling appeared in the ependyma and in the periventricular grey lining the 3rd ventricle without distinction among seasons; pronounced staining was observed dorsally and ventrally, where the symmetrical habenular ganglia and the unpaired pre-othic and infundibular recesses are respectively located.

In the midbrain the immune positivity was faint and even absent from some specimens in any season.

In the encephalic districts lying behind no labeling was identifiable in any specimen.

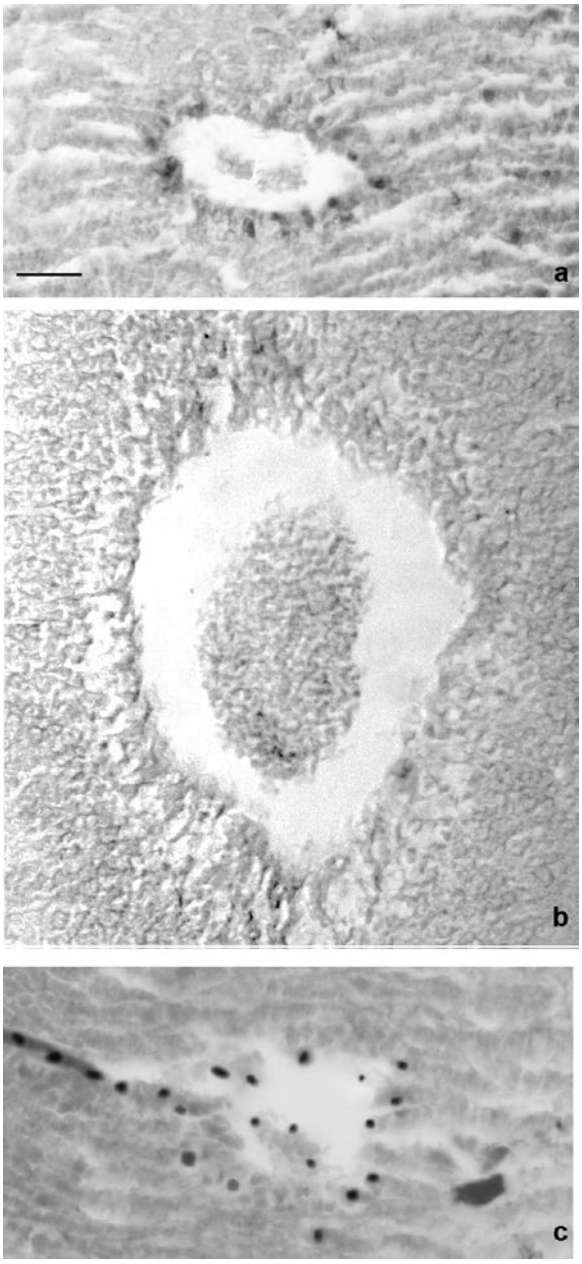

Fig. 1. Olfactory peduncles of normal adult *Podarcis sicula*. Labelling was mainly distributed in the layer lining the ventricles and rarely in the sub-ependyma. Scanty scattered PCNA-positive cells were found in specimens caught in late spring (Fig 1a), a more pronounced staining was present in specimens caught at the end of July (Fig. 1b) and numerous labeled cells were found in specimens caught in autumn. (Fig. 1c). [Figs. 1a, 1b: reprinted from Margotta, 2014a with permission]. Transverse sections. PCNA immunocytochemistry without nuclear counterstaining. Calibration bar=20 μm.

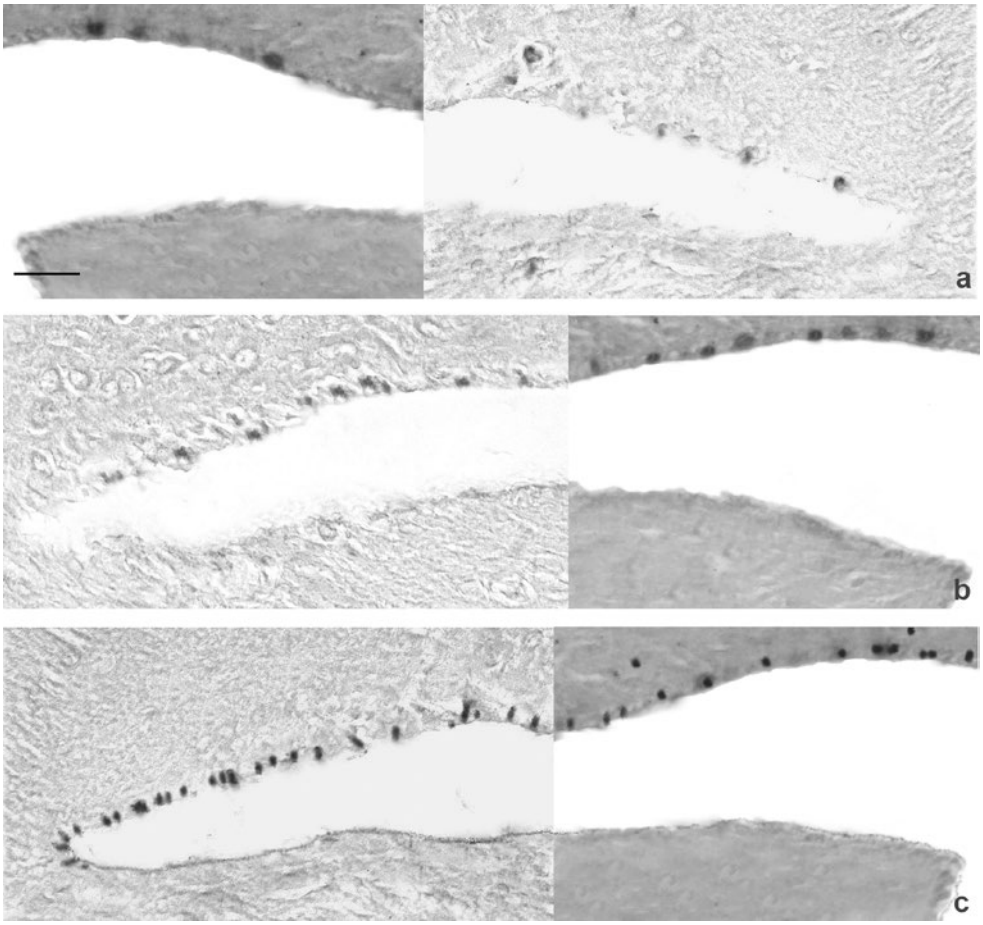

Fig. 2. Telencephalic hemisphere of normal adult *Podarcis sicula*. The immunolabeling was recognized in scattered ependymal cells and rare cells in the sub-ependymal layer in latero-dorsal and medio-dorsal position (*zonae germinativae latero-dorsales* and *zonae germinativae medio-dorsales* respectively), The labeled cells appeared increased from late spring (Fig. 2a) to the end of July (Fig. 2b) to autumn (Fig. 2c). [Figs. 2a, 2b: reprinted from Margotta, 2014a with permission]. Transverse sections. PCNA immunocytochemistry without nuclear counterstaining. Calibration bar = 20 µm.

Discussion

Many investigations support the opinion that cyclic fluctuations of seasonal environmental factors (temperature and photoperiod) could activate proliferation or impact on the natural or induced variations of proliferative potential in several tissues or organs of various systematic groups of heterothermal vertebrates, mainly Amphibians. That was ascertained for the cornea and eye lens (Rothstein et al., 1975), chemosensory epithelium (Dawley et al., 2000), retinal cells (Velasco et al., 2001), and the brain (Minelli et al., 1982; Bernocchi et al., 1990; Chetverukhin and Polenov 1993;

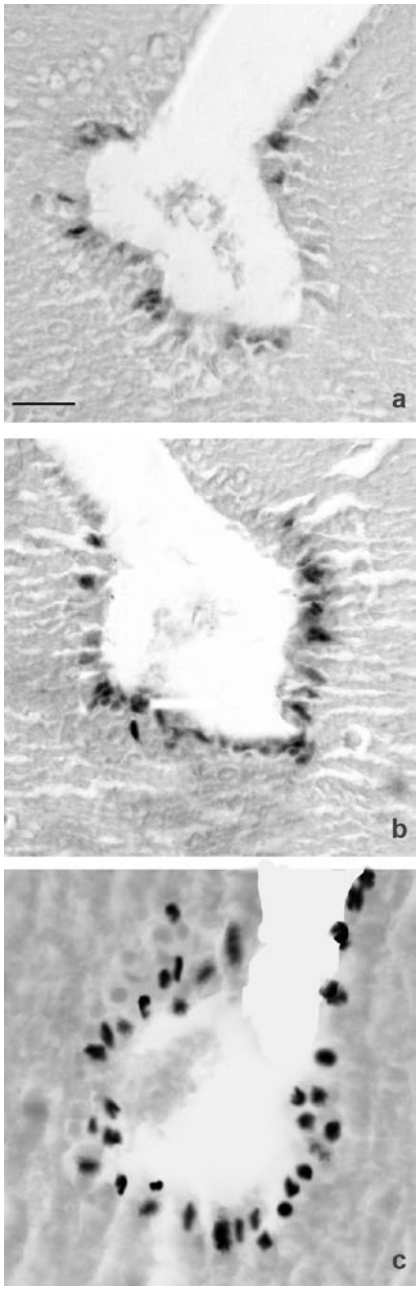

Fig. 3. Telencephaliç hemisphere of normal adult *Podarcis sicula*. The immunolabeling was recognized in sçattered ependymal cells and in the sub-ependyma layer in the vertical medio-ventral position (*zonae germinativae ventrales*). The labeled cells were increased from late spring (Fig. 3a) to the end of July (Fig. 3b) to autumn (Fig. 3c). [Figs. 3a, 3b: reprinted from Margotta, 2014a with permission]. Transverse sections. PCNA immunocytochemistry without nuclear counterstaining. Calibration bar = 20 µm

Polenov and Chetverukhin, 1993; Chieffi Baccari et al., 1994; Ramirez et al., 1997; Dawley et al., 2000; Vidal Pizarro et al., 2004; Margotta, 2012, 2014a; Margotta and Chimenti, 2016, 2017, 2018, 2019), These studies have also shown that one same season can stimulate different proliferative answers in different species.

In particular, for the brain the already mentioned papers of Minelli et al. (1982) and Ramirez et al. (1997) on *R. esculenta* and *P. hispanica* respectivey, must be exposed in detail.

Minelli et al. (1982) studied by autoradiography adult specimens of *R. esculenta* subjected to surgical injury and cold shock or to cold shock alone. The uptake of 6-H^3 thymidine in the brain showed a strict correlation between natural or artificial cooling and cerebral proliferative and regenerative potential. The mitotic activity and the regenerative capacity were low in May/June, became very high in September/October, declined in advanced November and reached a minimum at the beginning of winter. A temporary cooling (24 h at 4°C) led to an increase in proliferation in May/June and a decrease in September/October. The differences in mitotic rhythms along the year could explain the conflicting results reached by previous authors on the regenerative power of central nervous system in adult anuran Amphibians. Following previous studies (Rosomoff and Gilbert, 1955; Stone et al., 1956, Lougheed et al., 1960; Kiernan, 1979; Kiernan and Contestabile, 1980; Minelli and Del Grande, 1980), Minelli et al. (1982) proposed that a correlation exists between an influence of cold on blood brain barrier and regenerative capacity of brain tissue.

Ramirez et al. (1997) studied by autoradiography and immunohistochemistry adult specimens of *P. hispanica* subjected to experimental surgical injury and found a proliferative peak in summer. These authors also stated that cold (winter) temperature prevented migration of the newly generated immature neurons. This latter finding might hint to an influence of cold on radial glial cells, that are responsible for the translation of undifferentiated cells from the site of origin to the definitive place in the central nervous system in adult vertebrates (Margotta and Morelli, 1997).

A direct comparison between the report of Minelli et al. (1982) and that of Ramirez (1997) would be inappropriate because of relevant differences among species (in the sensitivity to seasonal conditions, experimental cooling and surgical injury) depending on the place in the evolutionary scale, habitat, timing of capture, laboratory environment and details of experimental procedures.

The physiological potential for proliferation of the uninjured, unstressed adult brain has been the subject of research in *P. sicula* (Margotta et al. 1999, 2005; Margotta, 2014a), *R. esculenta* (Margotta et al. 2000, 2005), *R. bergeri* (Margotta, 2012; Margotta and Chimenti, 2016, 2017, 2018) and *T. carnifex* (Margotta and Chimenti, 2019).

On the basis of the present and previously published data it may be stated that in normal adult brain of *P. sicula* the labeling for PCNA, indicating cycling cells, is scanty in spring, evident in summer, more evident in autumn; mainly restricted to the forebrain. "Matrix cells" are identifiable in olfactory ventricular surfaces, while "matrix areas" are identifiable in the telencephalic hemispheres as *zonae germinativae latero-dorsales, medio-dorsales* and *ventrales*. In the diencephalon labeling is found in the ventricular ependyma and periventricular grey matter, besides scattered labeled cells there are in the epithalamus, habenular ganglia, hypothalamic (infundibular and pre-optic) recesses. No labeling was seen in encephalic districts lying more behind.

Also the present observations in lizards seem to support and reaffirm some remarks, originated from previous investigations carried out in frogs (Margotta and Chimenti, 2017, 2018) and newts (Margotta and Chimenti, 2019), that the entity of the spontaneous proliferation seems too low to explain the reparative or even regenerative processes, obtained sometimes in that species by previous authors, reason why these processes could also rather depend on stimulation by the various (surgical, traumatic, thermal) experimental stimuli.

Autumn condition seems to remind a similar positivity exerted by an transient cold shock on brain proliferation in adult lizard (Margotta, 2014b).

The autumnal actual findings complete those previously reported for the same *P. sicula* caught in spring (Margotta et al., 1999, 2005) and summer (Margotta, 2014), showing an increasing trend in spontaneous proliferation of putative brain stem cells from springtime to autumn. We didn't make observations on winter specimens, assuming that those of Minelli et al. (1982) in *R. esculenta* and above all of Ramirez et al. (1997) in P. *hispanica* could hold also for *P. sicula*.

The overall moderate entitiy of the proliferative answer to environmental inputs, observed here, is in line with the diffuse awareness of a relative low regenerative potential of adult lacertilian Reptiles among poikilothermal vertebrates.

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Comparative anatomy

Plasticity of brain in normal adult some earth-dwelling Anamia and terrestrial Amniota: further review on the trend of seasonal environmental influence on encephalic proliferation, as revealed by immunohistochemistry

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Abstract

Further immunohistochemical evidence have been provided by the present authors about the persistence of latent natural proliferative potentially in adult brain of some low vertebrates and its response to cyclic seasonal environmental fluctuations (temperature, photoperiod) has been reviewed. These stimuli elicit an otherwise hidden mitotic activity thanks to stem cells still present especially in less high vertebrates like *Triturus carnifex*, *Rana bergeri*, *Podarcis sicula*. The evidence gathered from specimens caught in the wild in spring, summer and autumn has been compared with previous evidence on specimens of newts, frogs and lizards. Signs of proliferation were mainly observed in the typical sites (olfactory bulbs/peduncles, telencephalic hemispheres) occupied by cells in mitotic stand-by. The findings have shown increasing labelling from spring to summer to autumn with minor differences among species, and have confirmed that in adulthood the proliferative therefore the reparative and even the regenerative power of brain cells is highest in urodela (the vertebrates best equipped with quiescent cells), intermediate in anura and lowest in lacertilian Reptiles.

Keywords

Neural stem cells, matrix cells, matrixareas, Amphibians, lacertilian Reptiles.

Review

Since about a half century detailed awareness has been acquired on the plasticity of the brain in some adult heterothermic vertebrates: fresh water, earth-dwelling Anamnia and terrestrial Amniota (Margotta and Morelli, 1996).

Among these investigations a thread of research has been devoted to study if in adults the impact of cyclic seasonal environmental fluctuations (consisting in temperature and photoperiod variations) could exert any influence on brain in terms of physiological cell proliferative answer, or unmask a latent spontaneous proliferative power thus making apparent reparative and even regenerative potentialities due to an otherwise hidden mitotic activity of stem cells still present in the adult brain of some vertebrate species, mainly in lower ones.

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Such events have been correlated with the persistence in the adult of a stock of brain stem cells which have some own characteristics: morphological (being small and basophilic), physiological (able to proliferate), behavioural (persistence of the tendency to proliferate).

Usually these undifferentiated cells appear as scattered ("matrix areas"), as clusters of grouped cells, sometimes layered, in circumscribed areas ("matrix areas"), once nicknamed *Matrixzonen* (according to Kirsche, 1967) typically located among the ependymal cells lining each encephalic cavity and in the sub-ependymal layer, or elsewhere in cerebral tissues.

The number and size of such stem cells can vary among the different vertebrate groups and species; generally speaking, they appear much more numerous and large in lower than in higher species. In a gradual scale of value regarding the adult fresh water, earth-dwelling Anamnia and heterothermic terrestrial Amniota, such cells in mitotic stand-by appear to be relatively abundant in the urodelan Amphibians (the best provided with these cells among vertebrates), intermediate in the Teleosts, and relatively scarce in the anuran Amphibians and above all in the lacertilian Reptiles.

The number of such sleeping cells can vary according to different encephalic districts and is persistently high in the forebrain (olfactory bulbs/peduncles , telencephalic hemispheres), where they are mainly distributed in characteristic, mirror-like sites: the matrix cells in proximity of the olfactory cavities, the matrix areas at the edge, latero-dorsally and ventrally at the bottom of the sickle-shape, hallow ventricular surface of each telencephalic hemisphere: *zonae germinativae dorsales* and *ventrales*, respectively. In particular, only in lacertilian Reptiles each *zona germinativa dorsalis* appear sub-divisible in two portions, *lateralis* and *medialis*, like in *Lacerta viridis* (Minelli and Del Grande, 1980).

The *zonae ventrales* are the best provided with undifferentiated cells; intra-specific difference can be found among the brain of the earth-dwelling Anamnia and poikilothermal Amniota studied. Matrix cells can be observed here and there in the diencephalon. Such cells appear absent from the midbrain, in the *truncus cerebri*, and the *cerebellum* (in both sites, with the only exception of Teleosts in which it is possible to recognize symmetrical, additional areas provided with proliferative potential: the *zonae germinativae caudales* in the midbrain and scattered cells in the cerebellar deep tissue. Sometimes mitotically dormant cells can be found in the *medulla oblongata*.

Both telencephalic *zonae germinativae* are extended antero-posteriourly and active with different time courses: the *zonae dorsales* appear to exhaust their self-maintaining potential earlier than the *zonae ventrales*, which are generally wider and richer in cells (Kirsche, 1967).

All these information has been acquired and expanded through observations on untreated animals and upon experimental intervention, more frequently represented by brain surgery, ablations of encephalic plugs or wider portions sometimes with subsequent hetero- and rarely homo-transplatation (even of the whole brain), and *in vitro* culture of cerebral tissues. Analytical techniques were at first traditional histology, then autoradiography and immunohistochemistry, seldom electron microscopy.

These quiescent cells are remnants of the neural layer which forms in the early embryo and responsibles for the morphogenesis of the central nervous system (Kahle, 1951; Fujita, 1963; Kirsche, 1967), which may explain why the number of such cells decreases during the life of the organism: going from earlier to more advanced

embryonic stages, then through the subsequent larval ones - if present – and eventually into adult life.

The greater part of the information on this subject is the consequence of the exhaustive studies of Kirsche (1967, 1983), who investigated adult non-mammalian vertebrates by traditional histological methods. The studied species ranged from Teleosts to Birds, passing through urodelan and anuran Amphibia and lacertilian Reptiles. This author must be credited for the generation of most data on the features and localization of these putative precursor or stem cells in the adult brain and the recognition that they can be normally silent but are cable of self-reproduction and can start cycling again giving rise to descendants which undergo differentiation into neuronal or glial cells (Kirsche, 1967, 1983).

The persistence also in adult life of such cells in mitotic stand-by, thanks to their proliferative power, sustains both the physiological and experimental proliferative events and explains the reparative and even regenerative potentialities still present in the adult brain of many vertebrate species, especially in the less high ones.

In the last years investigations have been carried on adults of some earth-dwelling Anamnia (frogs and newts) and terrestrial Amniota (lizards) to investigate the spontaneous brain proliferative answer to cyclic seasonal environmental changes (temperature and photoperiod variations). These investigations were performed mainly by us with the help of immunohistochemistry for Proliferating Cell Nuclear Antigen (PCNA), expressed by proliferating cells (Miyachi et al., 1978; for further details on this method see Margotta and Chimenti, 2016).

By other authors in the past and by ourselves (Margotta, 2012, 2014a; Margotta and Chimenti, 2016, 2017, 2018, 2019a, 2019b) attention was paid to spring, summer and autumnal seasonal environment, while no attention was devoted to winter season conditions. In the last conditions autoradiographic studies were done by Minelli et al. (1982) on injured and uninjured brain of adult *Rana esculenta*, observing a post-autumnal arrest of encephalic proliferation that reached a nadir in full winter.

Immunohistochemical studies were done by Ramirez et al. (1997) on adult brain-damaged *Podarcis hispanica*, who referred that "…cold (winter) temperature prevented migration of the newly generated immature neurons".

An explanation on the "winter" outcomes both of Minelli et al. (1982) and Ramirez et al. (1997) could be found in a report by Margotta and Morelli (1997) which on the basis of the literature sources, furnished the matter of a critical review of the past acquired data on the analyzed relationships between contribution and persistence of radial glial cells in physiological neurogenesis and in post-traumatic conditions, advancing a hypothesis on the role of the glial cells in the plasticity of the central nervous system in the adults of several vertebrate species. Peraps, this information could to clarify and to justified the previous "winter" findings of Minelli et al. (1982) and Ramirez et al. (1997).

Therefore, was supposed that the different seasonal inputs on the proliferative rhytms could elucidate the controversial results reached by some authors in previous researches on the regenerative capacities of the central nervous system in the adult anurans.

Therefore, adult normal brain of *R. bergeri*, *Triturus carnifex*, *Podarcis sicula* – once, respectively, *R. esculenta* (Capula, 2000 a) *T. cristatus carnifex* (Bonifazi, 2000) and *L. viridis* (Capula, 2000 b) – were again investigated by Margotta (2012, 2014a), Chimenti and Margotta (2015), Margotta and Chimenti (2016. 2017, 2018, 2019a). Stain-

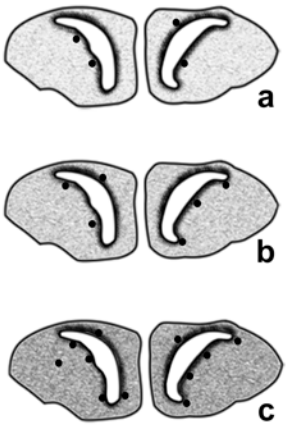

Fig. 1. Drawings (not to scale) of a transverse view of adult normal brain of *Triturus carnifex*. Olfactory bulbs in specimens caught in the wild in spring (a), summer (b), autumn (c). The dots represent PCNA immunolabelled matrix cells, isolated in the ependyma and periependymal grey matter.

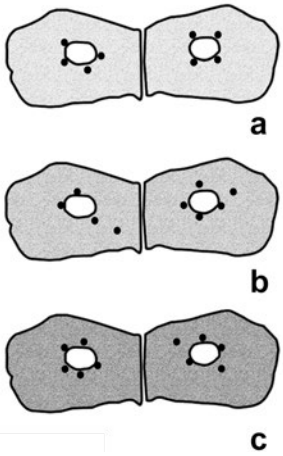

Fig. 2. Drawings (not to scale) of a transverse view of adult normal brain of *Rana bergeri*. Olfactory bulbs in specimens caught in the wild in spring (a), summer (b), autumn (c). The dots represent PCNA immunolabelled matrix cells, isolated in the ependyma and periependymal grey matter.

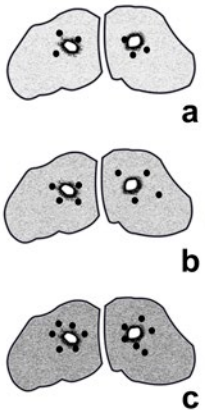

Fig. 3. Drawings (not to scale) of a transverse view of adult normal brain of *Podarcis sicula*. Olfactory bulbs in specimens caught in the wild in spring (a), summer (b), autumn (c). The dots represent PCNA immunolabelled matrix cells, isolated in the ependyma and periependymal grey matter.

ing was observed in the typical localization of putative stem cells, as "matrix cells" in the olfactory (Figs. 1; 2; 3) and diencephalic districts or as "matrix areas" in the telencephalic hemispheres (Figs. 4; 5; 6) where labelling was in correspondence of the ventricular edges of *zonae germinativae dorsales* (Figs. 4; 5), which in lizards are subdivided, as already said, each in *lateralis* and *medialis* (Fig. 6), and also in relationship with the bottom of the ventricular cavities in *zonae germinativae ventrales* (Figs. 4; 5; 6). Labelled cells were found among the ependymal cells and in the sub-epedymal layer. This pattern emerged also from further immunohistochemical studies on the same earth-dwelling Anamnia (Margotta and Chimenti, 2016, 2017, 2018, 2019a) and terrestrial Amniota (Margotta and Chimenti, 2019b). The findings were more evident in *T. carnifex* (Figs. 1, 4), less so in *R. bergeri* (Figs. 2, 5) and least in *P. sicula* (Figs. 3, 6).

In the investigated species the immunohistochemical signs of proliferation show an ascending trend from spring (Figs. 1a, 2a, 3a, 4a, 5a, 6a), through summer (Figs. 1b, 2b, 3b, 4b, 5b, 6b), to autumn (Figs. 1c, 2c, 3c, 4c, 5c, 6c), with only minor differences linked to the position of each species in the zoological/evolutionary scale.

The present authors' findings in different species and conditions have expanded those of Minelli et al. (1982) and Ramirez et al. (1997) and strongly support the hypothesis proposed by present authors, that the entity of the spontaneous proliferative processes along seasons accounts for the reparative or even regenerative phenomena obtained by previous authors, the extent of which could depend on syner-

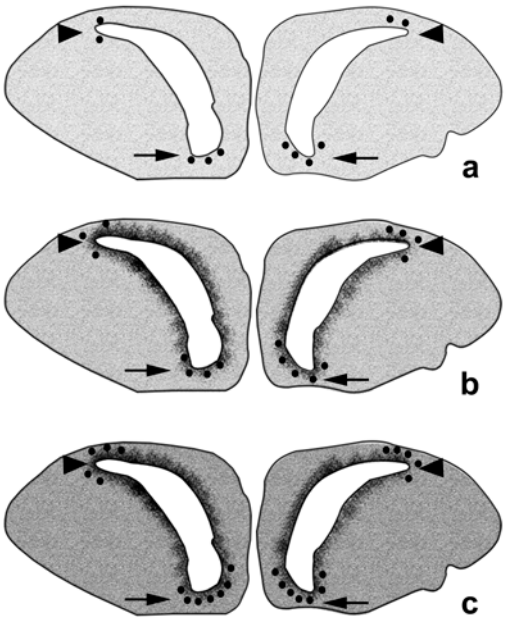

Fig. 4. Drawings (not to scale) of a transverse view of adult normal brain of *Triturus carnifex*. Telencephalic hemispheres in specimens caught in the wild in spring (a), summer (b), autumn (c). The dots represent PCNA immunolabelled matrix cells, clustered in the walls of telencephalic symmetrical ventricles to form the *zonae germinativae dorsales* (arrowheads) and *ventrales* (arrows).

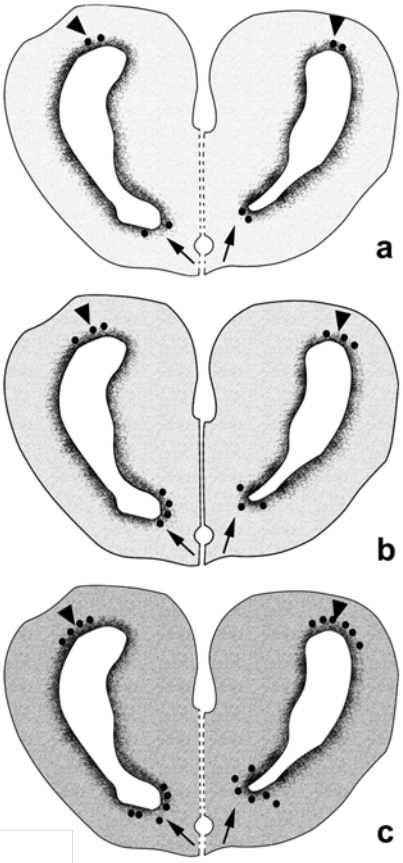

Fig. 5. Drawings (not to scale) of a transverse view of adult normal brain of *Rana bergeri*. Telencephalic hemispheres in specimens caught in the wild in spring (a), summer (b), autumn (c). The dots represent PCNA immunolabelled matrix cells, clustered in the walls of telencephalic symmetrical ventricles to form the *zonae germinativae dorsales* (arrowheads) and *ventrales* (arrows).

gic stimulation by various stressful stimuli: extreme temperature, surgery, other types of trauma. This was also supported by a comparison between the findings of previous authors on adult *R. esculenta* in autumn (Minelli et al. 1982) and on adult *P. hispanica* in summer (Ramirez et al. 1997) and those of present authors on uninjured adult *R. bergeri* (Margotta, 2012; Margotta and Chimenti, 2017, 2018), *P. sicula* (Margotta, 2014a; Margotta and Chimenti, 2019b), *T. carnifex* (Margotta and Chimenti, 2019a) and on adult poikilothermal earth-dwelling and terrestrial vertebrates subjected to cold shock (Chimenti and Margotta, 2013, 2015; Margotta, 2014b, 2015).

It can be said, therefore, that in adults the proliferative and therefore the reparative and even the regenerative power of the brain increases progressively from the lacertilian Reptiles to anuran Amphibians, Teleosts and eventually urodelan Amphibians, due to the respective, available stock of cells in mitotic stand-by of which the brain is espe-

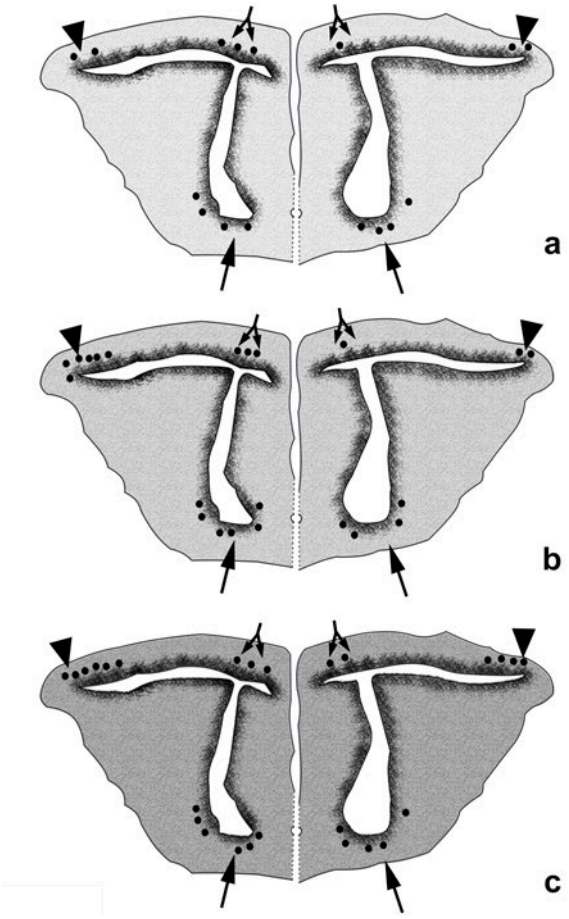

Fig. 6. Drawings (not to scale) of a transverse view of adult normal brain of *Podarcis sicula*. Telencephalic hemispheres in specimens caught in the wild in spring (a), summer (b), autumn (c). The dots represent PCNA immunolabelled matrix cells, clustered in the walls of telencephalic symmetrical ventricles to form the *zonae germinativae latero-dorsales* (arrowheads), *medio-dorsales* (arrowforkeds) and *ventrales* (arrows).

cially equipped in urodelans. The latter group of Amphibians is, among vertebrates, that which occupies the most privileged position, since these animals are the richest in undifferentiated cells, therefore those most endowed with proliferative potentiality and hence the best equipped for reparative or even regenerative processes.

Recently, in an immunohistochemistry report we exposed the influence of seasonal variations alone or coupled with an induced cold shock on spontaneous brain proliferation in adult poikilothermal earth-dwelling Anamnia and terrestrial Amniota; similar results were found in frogs, newts and lizards (Margotta and Chimenti, 2016).

Also now appear reasonable, as formely according to an our past opinion: is unthinkable that the spontaneous glimpsed proliferative phenomena owing to a seasonal input, referred in the actual report, could be able by oneself to justify the entity of the reparative or even the regenerative processes referred as obtained by previous authors. the degree of which could be explained attaching a stimulating value to further experimental various (thermal, surgical, traumatic) stressful stimulus, subjected by the employed samples.

The content of the actual exposition forms with that of the previous reports an *unicum* with which we have attempted to furnish a whole seasonal panorama of the natural cell proliferative trend as consequence of the impact of the cyclic seasonal environmental fluctuations (made of temperature and photoperiodic variations), revealed by the immunoreactive patterns, in the adult brain of heterothermic some vertebrates.

So we have satisfied an our recent wish: take cognizance of the overall annual view of the interrelations among such behavioural stimulus on adult encephalic cell proliferations in some earth-dwelling Anamnia and poikilothermal Amniota.

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History of Medicine

Michelangelo's David: triumph of perfection or perfect combination of variation and disproportions? A human perspective

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Abstract

Michelangelo's marble sculpture of David is one of the most admired works of art in the world. It is the most iconic symbol of the Florentine Renaissance, and a representation of the idealized human form in its perfection and proportion. The statue was examined in 2004 by two anatomists who observed the apparent absence of a single muscle. Our re-examination of the statue, from our perspective as clinical geneticists, shows unexpected and hitherto unpublished details of variations and disproportions within the overall context of exceptional harmony and beauty. This apparent contradiction raises the question as to what is considered to be morphologically "normal" and what "is not".

Keywords

Michelangelo's David, morphology, minor anomalies, reverse phenotyping, NGS.

Introduction

In Florence in 1501, Michelangelo Buonarroti (1475-1564) began shaping the statue of David from a block of marble initially discarded by the workers of the Opera di Santa Maria del Fiore, the cathedral of Florence. The block of marble was given, in 1463, to Agostino di Duccio for the realization of a colossal statue, perhaps a prophet or David himself (DeTolnay, 1933). The statue was meant to be placed on one of the buttresses of the Florentine Cathedral (the Duomo), as from a document of the Opera dating august 18, 1464 (Poggi, 1909). Since Agostino did not complete the work, the marble was given to Antonio Rossellino on may 6, 1476, but it was abandoned in the courtyard of the Opera till August 16, 1501, when it was taken by Michelangelo, and, by that time, the idea of placing it on the top of the cathedral had almost been given up (Falletti, 2002; Goldscheider, 2004; Paolucci, 2004; Acidini Luchinat, 2010).

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After having tested its consistency, Michelangelo, on October 14, ordered to built the "cage" of wooden board to work inside. On January 25, 1504, the statue was almost done, but the final decision on its placement had not yet been taken. Thus, a committee, including artists such as Andrea della Robbia, Cosimo Rosselli, Francesco Granacci, Piero di Cosimo, Davide Ghirlandaio, Simone del Pollaiolo, Filippino Lippi, Sandro Botticelli, Antonio e Giuliano da Sangallo, Andrea Sansovino, Pietro Perugino, Lorenzo di Credi, was appointed to reach a decision (Goldscheider, 2004). The Herald of the Signoria proposed to place the statue next to the door of Palazzo Vecchio, in the so called "Arengario", resuming the will of Michelangelo; whereas Giuliano da Sangallo was pushing the proposal of Leonardo da Vinci to place the statue inside the "loggia dell'Orcagna", within a black niche. The final decision was that of placing the statue next to the door of Palazzo Vecchio, on the site of the "Giuditta" by Donatello, which, on june 8, 1504, was placed inside the "Loggia dell'Orcagna". Simone del Pollaiolo and Antonio da Sangallo took care of moving the David, that, eventually, on September 8, 1504, was placed in the "Arengario" where stood up for the next three centuries (today replaced by a copy) (Landucci, 1883), and "nè mai più si è veduto un posamento sì dolce, nè grazia che tal cosa pareggi" (Vasari, 1906) ("never again has anyone seen such a beautiful and graceful masterpiece"). One of the absolute masterpieces of all time, the biblical hero stood as the perfect representation of the city of Florence; small compared to the giants of the large states of Europe, but capable of prevailing and winning with his strength and intelligence. In an epoch in which mass communication did not exist, the paintings and especially the statues situated in the city, were meant to transmit ideas and messages. Those of David are extraordinarily effective; it is a small, young, firm, determined, and winning hero. Several acts of vandalism and/or damage, caused by accident, characterized its history until the final placement in the Galleria dell'Accademia (Gallery of the Academy) of Florence, in 1872; where it is still possible to admire it today, after restoration (Figs. 1 and 2). Presently, we are lucky, as we can enjoy two copies of this masterpiece; a marble one, located in Piazza della Signoria, and a bronze one located in Piazzale Michelangelo, the terrace overlooking Florence.

Materials and methods

The statue was studied directly in the museum, thanks to the help and willingness of the then director Franca Falletti, and of professor Umberto Baldini. The measurements were made using rigid metric rulers, and measuring tape. The statue has a height of 516 cm, a weight of 5,660 kg, and a surface area of 19.47 square meters. It represents a 180-185 cm tall Caucasian male of the apparent age of 20-30 years. The actual measurements are illustrated in table 1. Of note, the traditional points of anatomical reference are accurately represented, allowing a detailed measurement of their reciprocal distances, which had been taken into account by that expert anatomist of Michelangelo (Premuda, 1957; Baldini, 1981, 2001; Condivi, 1998; Parronchi, 2003). The head is one eighth of the height. The occipito-frontal diameter measures 66.15 cm, versus the bi-parietal diameter of 43.70 cm, with a cephalic index (CI) of 66.06 (normal CI is between 76-80.9) (Gripp et al., 2013). It is important to keep in mind that when measuring the OFC in the live individual, we pay attention to the

Fig. 1. Frontal view of Michelangelo's David.

Fig. 2. Rear view of Michelangelo's David.

possible presence of thick hair, in order to avoid taking falsely elevated values. This has not been possible with the David, whose scalp hair appears thick and curly. The outer ears are of normal conformation, but the Darwin tubercle is not evident (Fig. 3). They have an angle of rotation of 22° degrees (the average value is usually between 17 and 22 degrees) (Gripp et al., 2013) (Fig. 4). The orbital region presents a distinct feature. The distance between the outer corner and inner corner of the right eye (5.6 cm), between the inner corner of both eyes (5.09 cm), and from the inner corner to the outer corner of the left eye (6.5 cm) does not respect the perfect symmetry of the "three eyes" (Aase, 1990). The left pupil appears deviated sideways as per a mild divergent strabismus. The lips are everted and the bilateral axes are perpendicular to both alae nasi, passing slightly through the corners of the mouth (Fig. 5), which is narrow. The chin is slightly prominent but rounded. The thorax is slightly inclined. A "groove" is recognizable between the spine and the right scapula; mimicking a pos-

Table 1. Measurements made in the Gallery of the Academy of Florence, by Massimo Gulisano and Pietro Antonio Bernabei, in 2004

Chin – vertex	69,83 cm
Facial Height	55,51 cm
Head Width	43,70 cm
Head Length	66,15 cm
Cephalic Index	66,06 cm
Bizygomatic Distance	33,54 cm
Biacromial Distance	123,54 cm
Bi-iliac Distance	87,83 cm
Penile Length	15,31 cm
Torso Length	135,17 cm
VII cervical vertebrae-coccyx	157,27 cm
Upper Arm Length	108,37 cm
Forearm Length	72,39 cm
Palm Length	32,17 cm
I Phalanx of the middle finger	15,78 cm
II + III Phalanx of the middle finger	15,16 cm
Palm Width	27,72 cm
Upper Leg	102,30 cm
Lower Leg	101,96 cm
Foot Length	77,75 cm
Foot Width	32,90 cm

sible hypoplasia of the extensor muscle of the spine (Fig. 6). The arm span is 611.28 vs. the height of 516 cm. The span based on a man's height of 182.5 cm, should be 216 cm. Therefore, the span exceeds the height by at least 33.5 cm. (Fig. 7). The ratio between the proximal and distal half of the body is 1.1, when it should be less than 1 for a male over 10 years of age.

The hands show visible dorsal veins on the right and not on the left, related to the limb posture; and the tip of the right middle finger (partly flexed) is close to the knee, appearing too long (Figs. 1, and 8). The hands measure 63.11 cm and the feet 77.75 cm. The ratio of the hands and feet to the median stature (estimated as 180-185 cm), corresponds to 22.32 cm and 27 cm, respectively. Regarding the genitalia, the testes appear proportionate and of apparent normal volume for an adult male (G5 Tanner), whereas the shaft corresponds more to that of an adolescent state (G3 Tanner), with a constriction of the glans as seen in phimosis. The David is standing on his right foot, with the body slightly swiveled to the right, the left leg slightly forward and flexed at the knee, the heel lifted from the ground, the left side of pelvis tilted up, the upper body slightly turned and flexed to right, the head partly turned to left (Fig. 1,

Fig. 3. The left ear of David.

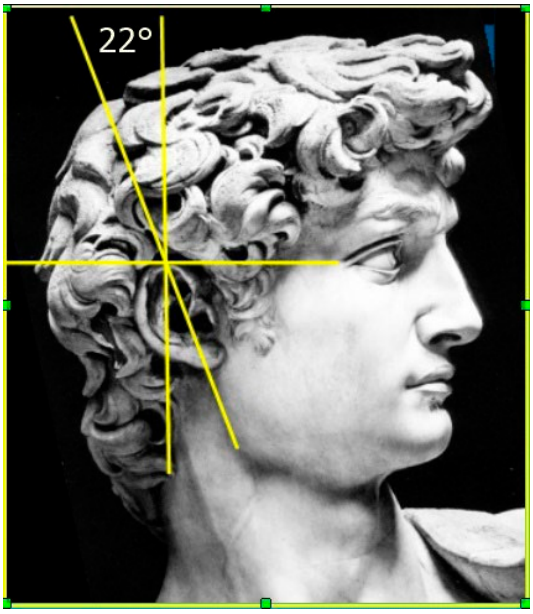

Fig. 4. Lateral view of David's head and right ear angle.

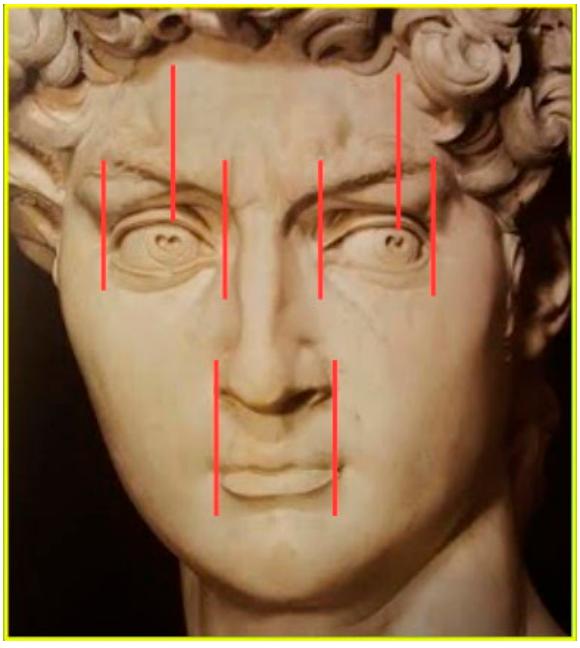

Fig. 5. The face of David.

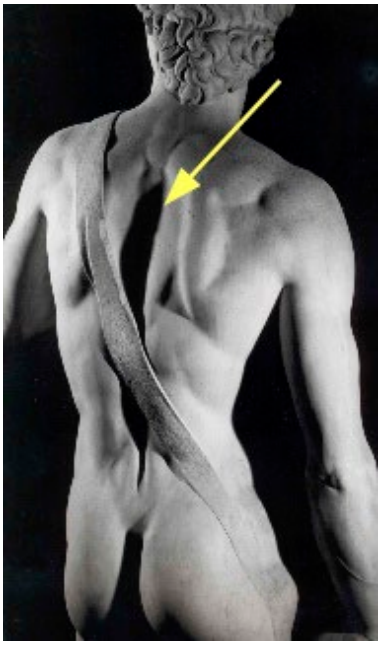

Fig. 6. The groove of the "missing muscle" on David's back.

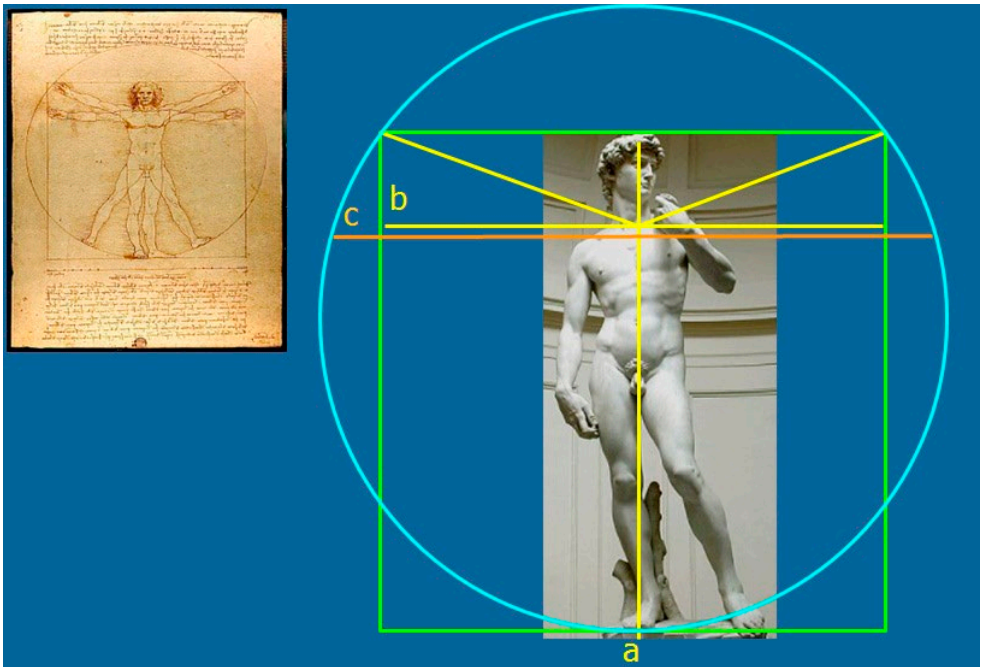

Fig. 7. a. Vertical yellow line: David's height. --b. Horizontal yellow line: Arm SPAN, cm 182.5 (as expected in relation to the height). --c. Horizontal orange line: Actual Arm SPAN (cm 216).

Fig. 8. "Physiological asymmetry".

Fig. 9. Middle panel; frontal view of David's face. Left panel: "Artificial" face constructed out of two right halves of David's face. Right panel: "Artificial" face constructed out two left halves of David's face.

and 2). In summary, the most relevant data demonstrate a "hyper"- dolichocephalic cranium, an arm span much greater than the height, an apparent unilateral extensor spinal muscle hypoplasia, and the upper section of the body almost the same as the lower one.

Discussion

The concept of the ideal human figure is credited to the Greek sculptor Polycletus, the elder, who promoted the pursuit of the theoretical model of the supreme ideal of masculine and feminine symmetry, balanced in its parts (Giuliano, 1998). Over the centuries the word "proportion" was synonymous with the "golden section" or "golden ratio", a concept that Leonardo called the "divine proportion" (Pacioli, 2010)) In the third book of the treatise "De Architectura" (1990), Vitruvio describes the ideal measurements of the face and the human body, and well known is its representation of the human figure inscribed in a square and a circle (Kruft, 1988). In this context the David of Michelangelo immediately impressed as a masterpiece of extraordinary beauty; acknowledged as an example of perfection for the form of the naked body, and for the masterly interplay of apparent proportions among the different body segments. The observance of the proportions in such a big statue suggests that Michelangelo started his work identifying the points of anatomical reference, always rigorously located. However, our examination and detailed measurements of the statue, while simultaneously confirming it as a wonderful representation of the human body, showed details and characteristics that do not fit a "perfect" model. Some of these, like the broad hands, may depend more on an optical impression (the view from the base of the statue) than a real disproportion, and, moreover, may have been intended for figurative purposes to indicate specific messages, such as strength and work. The "groove" on David's back has provoked more than a dispute on its interpretation. According to some art historians and anatomists, the "groove" might be due

to the absence of the extensor muscle of the spine, probably due to a defect in the block of marble upon which Michelangelo was determined to work (Vasari, 1906; Seymour, 1967; Baldini, 1981; Falletti, 2002; Goldscheider, 2004; Alvarez-Gonzales, 2008). However, based on Michelangelo's observations, it is not unlikely that he knew how this anatomical region shows great variability in the general population. Some authors in their treatise on human anatomy, note that there is a great deal of variation in the development of the spinalis muscles (Schaeffer,1893 and Kendall & Kendall McCreary, 2005). In addition, a recent British edition of Gray's Anatomy (1991) describes spinalis cervicis and capitis as variably blended and often absent. Of note, a recent study has reported the occurrence of an atrophy of the dorsal muscles, namely the infraspinous muscle, in professional pitchers (Pexa, 2017), whose sport is very similar to the slingsman movement (Friz, 2001). This might suggest that Michelangelo had observed such a muscle pathology frequently occurring in the slingsmen, rather than masquerading a defect in the marble. Last, but not least, the "groove" might also be explained by the dynamic posture of David, showing most of the weight on one leg, with the shoulders and arms twisted off-axis from the hips and legs. We should consider that the disproportions might correspond to the actual variations Michelangelo observed in the real world; it is well known that he and other artists of his time derived their models from the careful study of real bodies, and from their own environment (Pedretti, 2007). On several occasions, Michelangelo studied anatomy flaying cadavers to observe how the bones, muscles, nerves, and veins were tied together and would work in order to allow all the positions of the human body (Vasari, 1906). He could then be well aware of the existence of that variable physiological degree of asymmetry of the human body, known as "fluctuating asymmetry" (Opitz and Utkus, 2001), and David's face fully demonstrates it (Fig. 9). In addition, as has been done by other artists in the past, slight asymmetries or disproportions were deliberately sought in masterpieces (such as the bust of Nefertiti or the Venus Cnidia) to make the work more realistic. In the introduction of small imperfections a masterpiece gained dynamism and vitality (Bredekamp, 2016). Working at a time of spectacular rediscovery of classical statuary in Greece, Italy, Magna Graecia, Michelangelo was keenly aware of the artistic effects used by Phidias, Praxiteles, and their peers to impress, to elicit admiration, awe, and a keen feeling of aesthetic pleasure beholding their works. This involved the deliberate introduction into their sculptures of subtle asymmetries to heighten effects and to introduce that element of surprise and "interest" that distinguishes genius from mediocrity. On the other hand, does there exist a canon of perfection that counters our scientific concept of "malformation"? Or are there always common (minor) anomalies and variants of phenotype present even in the apparently "normal" human being? It is common knowledge that there exists an extraordinary and incredible variety of size and shape of the human beings as well as of all living species. Within this range of morphological diversity, the geneticist must be able to identify, describe, and distinguish: (a) the common variants (minor phenotypic variants or phenogenesis disorders with prevalence of >4%), (b) minor anomalies (phenogenesis disorders </=4%), and (c) malformations (embryogenesis disorders) (Merks et al., 2003). Accuracy in the recognition of the human phenotype and its precise description is becoming necessary as an indispensable resource for the current use of modern research technologies (Next-Generation Sequencing) that involve the understanding of new genetic conditions, and a correct

exchange of data with other doctors, laboratories, and researchers. An international group of clinicians (2009-2013) has worked to achieve a standardization of the terms used in the description of human morphology (Hennekam et al, 2013). In the same way, quantitative characteristics should all be carefully evaluated and measured with respect to the average within that ethnicity, and in that population by sex and age (Gripp et al, 2013).

Conclusions

Michelangelo's David is an artistic masterpiece of extraordinary beauty and of apparent correct proportions obtained through a perfect knowledge of the points of anatomical reference and of their ratio and reciprocal distances. However our aim is not that of an art criticism, which is far away from our expertise, but instead an analysis of the result in the light of what we can define a Michelangelo's "anatomical style" (Gulisano e Bernabei, 2001). It is well known that he employed the knowledge acquired by means of a long dissection practice to represent through the form, and, often even through its anomalies, precise symbolic and artistic concepts. On this purpose, let's think to the "Bacco" in whom he represented a mixture of masculine and feminine traits; or to the "Crocifisso di Santo Spirito" for which he used, as a model, the cadaver of a child to express the purity of Christ uncorrupted by the sin (Vasari, 1906; Condivi, 1998; Baldini, 2001). The unbelievable artistic level of the author allows that such anomalies contribute to the artistic value of the masterpiece. At this point, we wish to stress the following: 1) the points of anatomical reference are accurately described and represented throughout the David's body; 2) the layers of dissection are accurately respected, as we can appreciate in both hands; 3) the muscles are represented either in contraction or relaxation according to the movement that a particular body part is involved in; as comes from the observation of the peroneal muscles and, even better, from the muscles of the limb going to employ the sling, and from the mimic muscles (see the contraction of the procerus and of the nares dilatator muscle, and elevator of the lip, expressing aggressiveness). All this allows us to speak of a true Michelangelo's anatomical style. Therefore, we consider significant that our detailed examination of the statue revealed surprising quantitative and qualitative morphological variations, not just explained by the initial idea of placing it on one of the buttresses of the Florentine cathedral (the Duomo). In fact, when Michelangelo took care of the marble, that initial project had been given up, and his contemporaries state that he pushed so that its placement would be in the "Arengario", moving the "Giuditta" by Donatello (which was placed there), inside the "loggia dell'Orcagna" (Landucci, 1883; Vasari, 1906; Falletti, 2002; Goldscheider, 2004; Paolucci, 2004; Acidini Luchinat, 2010). The above seemingly contradicts the view of David as a masterpiece of absolute perfection from a strictly anthropological point of view. We could instead describe it as a masterpiece of a "perfect" combination of anomalies and common traits. This correlates with our daily experiences working with real world individuals; common variants and minor anomalies may always be present even in apparently normal and harmonic phenotypes. The role of the medical geneticist that seemed to be slowly and inexorably declining, finds itself today in a renewed and essential mission. Precise measurements are valuable and

necessary to adequately define phenotypes in order to diagnose, target, and correlate them with the identified genotypes. The challenges of the technological revolution in molecular genetics (NGS) make it even more essential to establish a correct phenotypic correlation to the genomic variants not yet described or of unknown significance. Among the many messages that the marvelous statue of David gives to humanity, we have also found one for medical geneticists; i.e., the ever-present morphological variations and disproportions demonstrating the extraordinary variability of phenotypes and the beauty of each ethnicity and of each living species. Of note, the anatomical, anthropological, and biological observation of Michelangelo was highly original for those times, when the basis for the knowledge of the human body were just founded by the artists. It was only later that physicians, such as Vesalio, carried on the study, but still exploiting the artistic knowledge, turning to great artists for the anatomical tables of their own books (see G.B. Morgagni with Tiziano; R. Colombo with Michelangelo) (Premuda, 1957, Ficarra, 1968, Singer, 1993, Condivi, 1998, Parronchi, 2003).

"To escape the world, there is no surer way than through Art, and there is nothing better than Art to keep one connected to it."
Johann Wolfgang Goethe (1992).

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Learning

The anatomical and historical background of surgery: major surgical achievements during the Middle Ages and the Renaissance

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Abstract

Anatomy constitutes the historical and epistemological background of surgery and surgery, in turn, is the area of medicine dealing with the management of injuries and pathologies by means of manual interventions and instrumental devices. As such, surgery may be considered as old as mankind. However, only in the Age of Enlightenment (eighteenth century) was the rigid and negative distinction typical of the past between clinical medicine and surgery overcome. This historical differentiation is by many historians of Western medicine ascribed to the famous Hippocratic Oath, a deontological text attributed to the Hippocratic School (V-IV centuries B.C.). The object of this contribution is the description of the evolution of surgery in the course of the Middle Ages and the Renaissance, periods in which a number of fundamental acquisitions in surgical practice were gained, ranging from a more correct treatment of wounds and lesions to the elaboration of the first effective methods for vessel ligature, from the improvement of amputation techniques to the refinement of trauma surgery, from the major progress in human anatomical knowledge to the invention of new surgical devices, including the obstetrical forceps. Last but not least, the achievement on the part of surgeons of a more codified professional role, their acquisition of a more honourable deontological profile and the definition of their clearer collocation in the sanitary panorama, appear as paramount historical-epistemological achievements typical of the surgery practiced during the Middle Ages and the Renaissance.

Keywords

Anatomy, surgery, history of medicine, middle ages, renaissance, therapy.

Anatomy constitutes the historical and epistemological background of surgery and surgery, in turn, is, according to the Encylopaedia Britannica, the "branch of medicine that is concerned with the treatment of injuries, diseases, and other disorders by manual and instrumental means" (Encyclopaedia Britannica, 2019). Since injuries are particularly common in children, and since manual practice has always been the first line of intervention to try to manage human alterations and disorders, surgery may be considered as old as mankind. Nevertheless, only in relatively recent times has the tight and negative distinction of the past between clinical medicine and surgery been overcome (Conti, 2011). This historical differentiation has by many historians of Western medicine been ascribed to the famous Hippocratic Oath, a "deon-

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tological" text attributed to the Hippocratic School (V-IV centuries B.C.) with the purpose of regulating the professional behaviour of the medical class in ancient Greek society (Encyclopaedia Britannica, 2019). In this ethical code, the following is written: "I (i.e. the physician) will not cut persons labouring under the stone, but will leave this to be done by men who are practitioners of this work" (Smith, 1979). Such a prescription literally means that it was neither effective nor safe to perform what today is called "major surgery" in patients. Independently from the technical ability of surgeons, operated people of the time would almost invariably have had a negative prognosis due to haemorrhage, pain and/or infections (Conti and Gensini, 2008), so that the Oath in practice invites the doctor not to undertake this commitment. Unfortunately, some interpreted this passage of the Hippocratic Oath as the documentation that surgeons were to be considered as minor characters if compared to physicians-clinicians, paving the way to a rigid, and inappropriate, separation active till the eighteenth century. However, in the four centuries immediately preceding this period a number of fundamental acquisitions in surgical practice were gained, and these achievements of the Middle Ages and the Renaissance are the object of this contribution.

Until the thirteenth century, the suppuration of wounds was considered a normal and even favourable process; as such it was not to be contrasted. Henri de Mondeville (c. 1260-1316) was among the first scholars, in the Middle Ages, to dispute this opinion, thus allowing the evolution of surgery (Grant, 1974). Commonly considered one of the fathers of French surgery, de Mondeville studied in Montpellier and in Paris. His text "Cyrurgia" ("Surgery", 1312) was one of the first complete treatises of surgery in Europe, surpassed only by the book "Chirurgia Magna" (1363) of Guy de Chauliac (c. 1300-1368) (Glick et al., 2005). Guy, one of the most important surgeons of the Middle Ages in the Western world, described narcotic inhalations for surgical patients, and his fundamental text remained a surgical reference manual for at least three centuries.

Giovanni da Vigo (1450-1525) is remembered for having been the first Italian surgeon to write a report on firearm lesions and their treatment. In his 1514 treatise "Practica in Arte Chirurgica Copiosa", consisting of nine books, da Vigo rendered available a complete picture of the European surgery of his time, testifying that the medication of gunshot wounds continued to remain a major problem in the Renaissance. This is also demonstrated by the number of infections and deaths recorded on sixteenth century battle-fields (Da Vigo, 1514; Gurunluoglu et al., 2003).

The French physician and surgeon Ambroise Paré (1510-1590) was a pioneer both in the treatment of wounds and in the field of the ligature of blood vessels. Paré, by many experts considered the father of modern surgery, while acting as barber-surgeon in the service of Colonel-General René de Montejan, finished the boiling oil commonly used to treat wounds during a battle. He therefore decided to adopt a light mixture of turpentine, rose oil and egg yolk, and observed an obvious improvement in the healing of the soldiers treated with this new intervention if compared to the wounded managed with the conventional burning system (Poirier, 2005). In his paramount text entitled "Méthode de traiter les plaies faites par les arquebuts et autres bastons à feu, et celles qui sont faites par la poudre à canon" (1545), he accurately described the ointment he had elaborated on the battle-field, and he successively refined it, empowering its emollient and cicatrisant actions (Doe, 1937).

Massive war wounds often forced military surgeons to amputate legs and arms. The availability and diffusion between the Middle Ages and the Renaissance of new and powerful firearms caused, on the one hand, a worsening in severity and frequency of lesions, but, on the other, contemporaneously prompted an improvement in amputation techniques on the battle-fields. The Renaissance improvement consisted in a higher speed in amputation (the best surgeons were considered those able to amputate a thigh in less than thirty seconds), in a larger removal of bone tissue while sparing as much as possible of soft tissues so as to guarantee future healing, and in an increased attention to vessel ligature methods. In the absence of antibiotics (discovered only in the twentieth century), surgeons could not efficaciously fight infections, but it was precisely in the sixteenth century that blood loss, one of the three major historical hurdles to the full implementation of surgery, started to be controlled (Conti and Gensini, 2008).

In the first half of the sixteenth century the study of anatomy, and consequently the practice of medicine and surgery, were revolutionized by the Flemish physician Andreas Vesalius (1514-1564). Following numerous personal dissections of cadavers, Vesalius prepared the first modern illustrated textbook of human anatomy, the "De humani corporis fabrica libri septem" (1543), still today retained to be one of the most influential books in the history of Western medicine (Vesalio, 1543). After more than a thousand years, this Belgian scientist questioned the anatomical system of the physician Galen of Pergamum (ca. 129-216), largely surpassed it and led anatomical study into the humanistic climate. Not by chance did Paré translate from Latin into French various chapters of the anatomical manual of Vesalius, including them in his anatomical-surgical treatise "Anatomie universelle du corps humain" (1561) in order to give ample diffusion to the new anatomical culture among French surgeons. A great merit of Vesalius was also that of claiming for the physician-surgeon a direct role in personally dissecting human bodies. In the Middle Ages, even during academic lessons of anatomy, this function had been exerted in a non-systematic way by non-graduated secondary figures, such as dissectors (Van Hee, 2016).

In the sixteenth century other branches of surgery underwent relevant evolution, including obstetrics, head injuries management and trauma surgery. In the field of obstetrics the surgeon Peter Chamberlen (1560-1631) should be remembered. Born in France, he studied in England where he became a famous "accoucheur" (obstetrician) and to him is nowadays attributed the elaboration of the first obstetrical forceps (Sheikh et al., 2013). With regard to ever present military surgery, the Royalist surgeon Richard Wiseman (1622-1676) practiced for a long time complex operations on the battle-fields and, when he returned to practice civilian general medicine in London, he transferred the notable technical skills he had acquired in war to everyday trauma surgery and to innovative treatments of head injuries (Hull, 1996). Considered by many historians the father of English surgery, in his celebrated book "Severall Chirurgicall Treatises" (1676) he furnished the descriptions of hundreds of patients personally treated in the course of the English Civil War (Wiseman, 2011).

To summarize, and in conclusion, many and extraordinary were the achievements in surgery in the course of the Middle Ages and the Renaissance. They ranged from a more correct treatment of wounds and in general of firearm lesions to the elaboration of the first effective methods for vessel ligature, from the improvement of amputation techniques to the refinement of trauma surgery, from the remarkable progress in

human anatomical knowledge to the invention of new surgical devices, including the obstetrical forceps (Temkin, 1951). Last but not least, the identification on the part of surgeons of a more codified professional role, their achievement of a more honourable deontological profile and the definition of their clearer collocation in the sanitary panorama, appear as the major historical acquisitions of surgery in the course of the Middle Ages and the Renaissance (Nutton, 1985).

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Case report

Social media Facebook and You Tube usefulness in anatomy learning: experience at Sapienza University of Rome

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Abstract

Digital natives are growing up in a new media ecosystem, where the use of the net and social media is a daily practice. Even if there is a growing interest in the use of social media in university education, there is a paucity of outcome based, empirical studies assessing the impact of social media in medical education, in particular in the Human anatomy field. To facilitate human anatomy learning and teacher-student relation, a Sapienza university of Rome human anatomy teacher (HAT) created a professional Facebook profile (HATPFP) and a You Tube channel dedicated to human anatomy topics (HATYTC). In order to assess the usefulness of social media not only in human anatomy learning but also to get in touch with the human anatomy teacher, at the end of each course a survey was created than distributed to the students of health professions and medicine and surgery degree courses. Our data, the first referring to the Italian context, show a useful and positive opinion by most students on the use of social media Facebook and You Tube in the teaching of Human Anatomy. Although within the limits of an exploratory study, we have highlighted how social media can be an effective support for anatomy teaching by facilitating social interactions (in terms of time reduction, simplification, immediacy, less formality), improving learning (in terms of memorization and understanding of concepts: and notions of anatomy), and making students autonomous in their search for new knowledge of anatomy.

Keywords

Anatomy learning, facebook, you tube, digital natives, social media, distance learning.

Introduction

The term *Digital Natives*, was coined originally by Marc Prensky (2001), to identify a new generation of humans born after 1990 (they were also called N*et-generation* from Oblinger & Oblinger 2005, and *Millennials* from Tapscott, 1997). Digital natives have never known a world without computers, internet, video games, and mobile phones (Roberts, 2005). Internet and related technologies have a major influence on digital native's culture and development, in fact they are growing up in a new media ecosystem, where the use of the net and social media is a daily practice. University

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students, for some years now, belong to the generation of digital natives, their life is often characterized by the time spent on public transport to reach the university, during the journey students use their smartphone both to communicate with each other and to take advantage of multimedia content and surf the Internet. The distances and traffic of a large city sometimes make it difficult for students to meet a professor, especially if the teacher's office is far from the one where the lessons are held or from the hospital where they attend the wards. Sudden meteorological or socio-political events can lead to the displacement of lessons or exams to another date or place, the timely notification of such changes is essential to avoid unnecessary loss of time for students. Studies in literature (Siemens, Weller 2011; Manca, Ranieri 2013) state that social media have shown to have potential as learning environments, if developed within educational projects. According to several authors, using social media and new learning platforms allows the research of increasingly rich and complex materials and stimulates the creation of virtual communities. Moreover they call for a collaborative, reflective and metacognitive approach to study through the comparison of the objectives and content of educational activity (Cacciamani & Giannadrea, 2004; Scardamalia & Bereiter; 2004; Sthal, Koschmann, & Suthers, 2006; De Marco and Albanese 2009; Trentin, 2011; Varisco, 2000, 2008). Although these surveys show a growing interest in the use of social media in university education, there is a paucity of outcome based, empirical studies assessing the impact of social media in medical education (Magro et al. 2013; Sutherland and Jalali, 2017; Fisher et al., 2018). This is more valid as far as the anatomy education is concerned, in fact the research so far about the use of social media in anatomy education is limited and lacks comparative studies (Cytas, 2019).

Aim

To facilitate human anatomy learning (allowing students to study also on public transport), and to be able to communicate with their students (belonging to various degree courses located in various locations in the Rome area), quickly and effectively, a Sapienza university of Rome human anatomy teacher (HAT) created a professional Facebook profile (HATPFP) and a You Tube channel dedicated to human anatomy topics (HATYTC).

In order to collect the opinions of students belonging to various degree courses on the usefulness of the two social media in human anatomy learning and to get in touch with the human anatomy teacher, at the end of the course year a survey was created than distributed to the students through the HATPFP.

Materials and methods

The main structure of the human anatomy courses

The anatomy course topics are in line with the Italian core syllabus recommendations and international suggestions (McHanwell et al., 2007; Drake et al., 2009). Health professions anatomy course takes place during twelve-week in the first semes-

ter of the degree course first year. Medicine and Surgery anatomy course takes place during three semesters in the first and second degree course years. In both Health professions and medicine and surgery degrees students attend to lectures, workshops and practical laboratory classes. During practical classes, students work in small groups under teaching tutors supervision, using cadaveric prosection, plastinated specimens, plastic models, plastic bones, radiographs, MRI and CTI scans (Familiari et al., 2013).

The human anatomy teacher facebook profile (HATPFP)

The HATPFP was created as well as anatomy lessons in various degree courses started. During the first lesson in each degree course, the HAT showed a slide with all contact details: room phone, e-mail, and name of PFP and YTC. HATPFP was used by the teacher to share useful information, such as the lesson calendar (with related topics, specifying time and location), dates and location of exams, the presence of scientific seminars of possible interest to the students, the deepening of some anatomy issues, as well as the reference to the You tube channel.

The human anatomy teacher you tube channel (HATYTC)

You tube allows the viewing of thematic multimedia movies, facilitating their organization by topics using the playlist tool. This is a great utility to collect in an organized and consistent way videos from different sources. It is also possible to add to the videos themselves keywords and labels, making research faster and more targeted. HAT has created playlists divided by subject, containing videos taken from some scientific channels such as Anatomié 3D Lyon, Agorà Biomedical Sciences, UCD Medicine, Nucleus Medical Media, Neuromatìq, Great Pacific Media and VMV3D (Vision Medica Virtual 3D), associated with other videos uploaded by personal channels of university teachers of various nationalities. The videos are mostly in English, but also in Italian, French and Spanish.

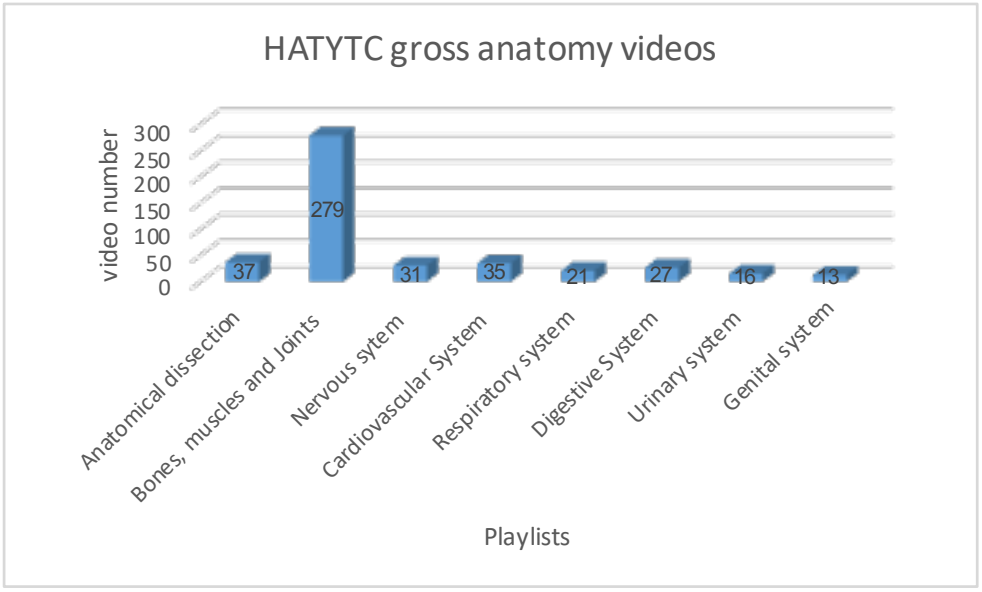
The You tube channel contains 459 human anatomy videos and 130 cytology, histology and biology videos. The videos are classified as reported in Graph 1 and 2.

Students and degree courses

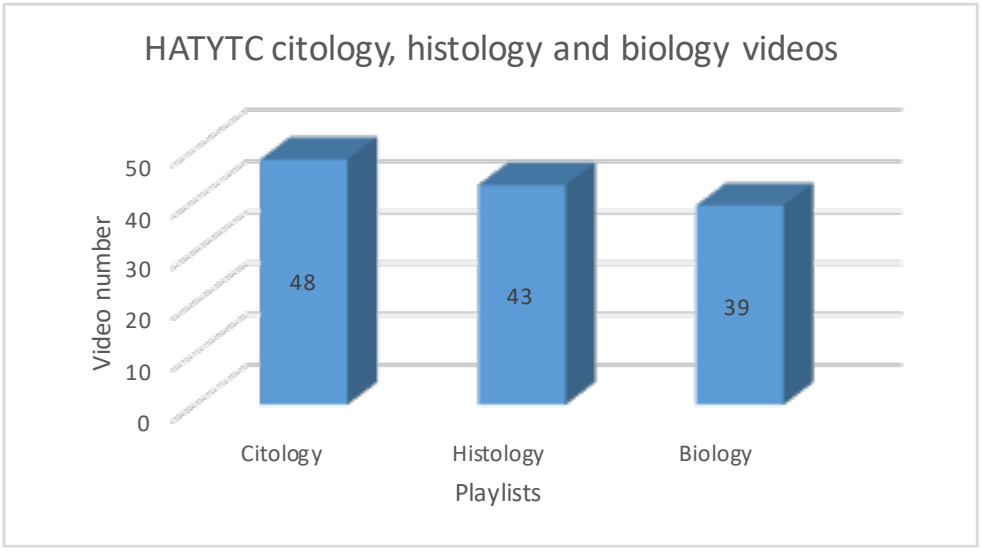
The students who participated in this survey belong to the following degree courses of the Faculty of Medicine and Psychology: Medicine and Surgery, Nursing, Obstetrics, Physiotherapy, Podiatry, Occupational Therapy, Orthopaedic Techniques, Psychiatric Rehabilitation Techniques. Their distribution is showed at graph 3.

The surveys

Two surveys were designed and then distributed. The first was a pilot survey and the second one was a multiple choice survey. The first one consisted in 21 questions, some with dichotomous answers and others with open answers, involving 25 students belonging to the Degree Courses in Medicine and Surgery. Students were recruited thorough a call on the HAPFP on voluntary basis. A qualitative content



Graph 1. Topics of thematic multimedia movies in the human anatomy teacher you tube channel (HATYTC).



Graph 2. The You tube channel also contains videos of biomedical basic sciences, such as cytology, histology and biology.

analysis (Graneheim and Lundman, 2004; Hsieh and Shannon, 2005) allowed us to identify the categories of answers to turn open-ended questions into closed-ended questions and select the most relevant questions to prepare a final multiple-choice

survey that would be distributed to a larger number of students. This second survey had an initial incipit (that constitutes the informed consent, in which the questionnaire is presented, the average filling in time is communicated, the privacy regulations are explained and a reference e-mail is provided for further questions or curiosities on the subject). The survey consisted of 15 multiple-choice questions divided into 5 sections. In the first section personal information were collected (age, gender and belonging degree course). The second section had explored the familiarity and use of a Facebook and You Tube account before enrolling at university. The third section concerned the use of HATPFP and the perception of its usefulness. The fourth section focused on HATYTC use and usefulness perception. The fifth section asks for an overall judgment of social media usefulness in anatomy learning.

The questionnaire was distributed as a Google Modules file to the students recruited by HATPFP.

Answers collection was between from June to October of the same academic year in which the students attended the lessons. Then the statistical analysis of the results was carried out.

Survey participation was anonymous, voluntary and free of charge. The Ethics and Research Committees of investigators Departments approved the research protocol of this study.

Results

First section: Students gender, age, attended degree course

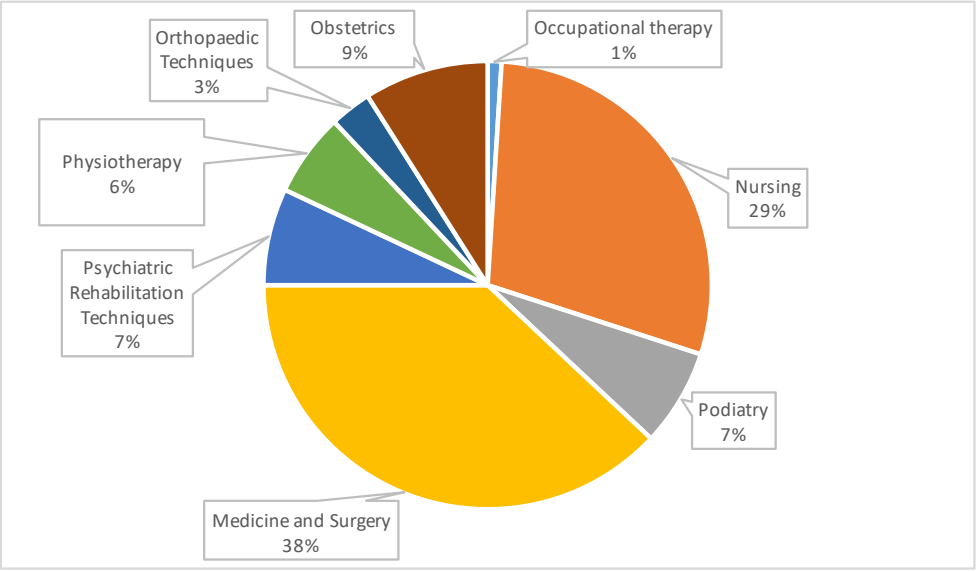
100 surveys were collected, 35% belong to males, 65% to females. Mean students age was 20,73±2,96 years. Most of the students who completed the questionnaires came from the courses of Medicine and Surgery (38%) and Nursing (29%). Students percentage for each degree course is listed in graph 3.

Second Section: Familiarity and use of a Facebook and You Tube account before enrolling at university

The results of the second section show that all the students examined belong to the generation of digital natives, and that the use of social media facebook and you tube is part of the common practices already in high school. In fact, 98% of students had a Facebook profile before entering university, and 99% of students declared the personal use of You Tube before entering university.

Third Section: The use of HATPFP and the perception of its usefulness

The results of the third section show that most of the students (84%) used HAPTFP, and the main motivation was (it was possible to choose more than one option) to have information about the examination modalities (68, 6%) followed by news about the videos published in the you tube channel (46,5%). The request for explanations on topics dealt with in class and the requests for material to deepen some chapters were placed on an equal footing (38.4%). The request for information on the integrated



Graph 3 Students attending degree courses.

course in general (23.3%) and information on seminars of possible interest to students (15.1%) were the options that received the least interest. 94% of students consider HATPFP to be a useful overall means of communication with the teacher, especially for its speed (75.8%), ease of use (63.7) and informal mode of communication (Table 1).

Fourth section: HATYTC use and usefulness perception

The results of the fourth section show that about two thirds of the students (71%) used HATYTC, of whom almost all (97%) say that HATYTC is a useful tool for learning anatomy. Analyzing the reasons for this opinion, it emerges that about half of the students (52%) believe that watching videos facilitates both the understanding and the memorization of anatomy. The understanding of the three-dimensional structure of the organs and the ability to visualize the concepts studied are around 17.4% and 15.3%, only for 13.3% of the videos implement the content of textbooks, while 2% indicate the other voice. It is very interesting to note that the use of HATYTC pushes students to autonomously search for other video material (63%) both of anatomy (49.2%) and of related subjects (39.7%) (Table 2).

Fifth section: Overall judgment

The fifth section asks the student to express an opinion on the usefulness of the two social media in the study of anatomy. HATYTC was the most voted (38%) followed by the statement Both (30%). HATPFO received 21% of the votes. Only 11% of students declared both social media useless in anatomy learning (Graph 4).

Table 1. The use of HATPFP and the perception of its usefulness

Did you use HATPFP?	Yes	84 %
	No	16%
If Yes, What did you use it for? (it was possible to select all applicable items)	To ask HAT for examination details	68,6%
	To request for explanations on topics dealt with in class	38,4%
	To get information about seminars	15,1%
	To get information about video published in the HATYTC	46,5%
	To acquire materials and files for deepening the lessons	38,4%
	To have explanations on some topics	23,3%
Do you think HATPFP is a useful way to communicate with the anatomy teacher?	Yes	94%
	No	6%
If yes, for what communicative characteristic? (it was possible to select all applicable items)	Fast communication	75,8%
	Informality of communication	31,9%
	Ease of use	63,7%

Discussion

The literature shows that university students have differing views on the educational usefulness of social media. In some studies the data reveal that students would prefer to use social networks (i.e. facebook) only for social purposes without attributing formal teaching purposes to them (Selwyn 2009; Madge et al. 2009; Wise et al., 2011). In others, the results are the opposite, because the greatest opportunities for sharing teaching materials, mutual support and relationship building are highlighted (Bosch 2009). According to Roybler et al. 2010, students are in fact more inclined to use Facebook alongside classroom work than teachers, who prefer the use of more traditional technologies such as the use of e-mail. Since in the literature there is no survey on the Italian reality, we wanted to verify the usefulness of HATPFP and a HATYTC in the study of human anatomy at some degree courses of the Faculty of Medicine and Psychology of the University La Sapienza in Rome.

Since both media are already used by students, we can say that HAT's use of HATPFP and HATYTC is not an unknown practice but rather fits into virtual habits and places already familiar to students. We therefore believe that linking teaching to communicative and cognitive practices already widespread among students can be very effective both in improving teacher-student communication and in improving

Table 2. HATYTC use and usefulness perception.

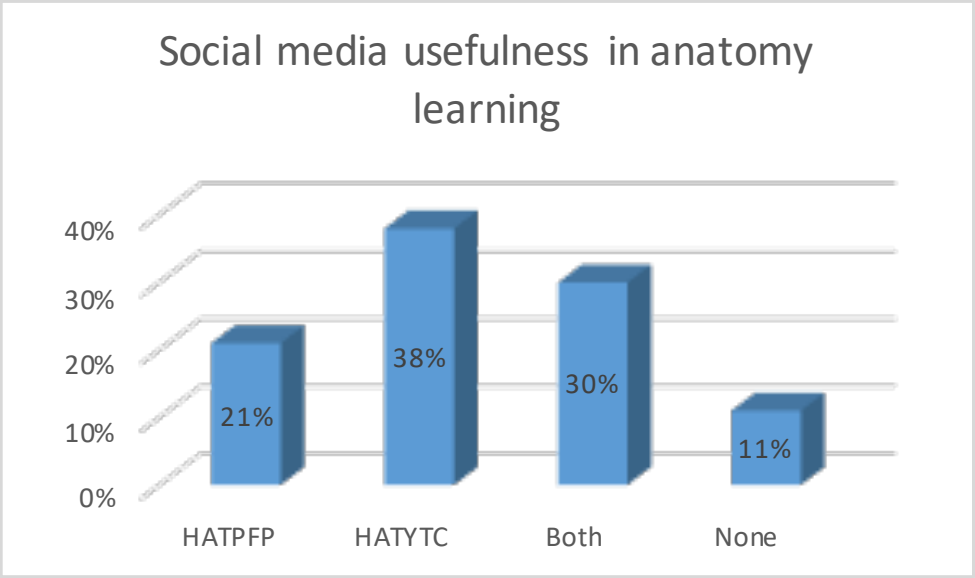
Did you use HATYTC?	Yes	71
	No	29%
Based on your experience, do you think that HATYTC is a useful tool to support anatomy learning?	Yes	97%
	No	3%
If yes, why? (choose only the answer that best reflects your opinion)	Because videos allow to easily visualize the studied concepts	15,3%
	Because videos implement textbook content	13,3%
	Because videos allow to understand the three-dimensional relationships between organs	17,4%
	Because videos facilitates anatomy understanding and memorization	52%
	Other:	2%
After viewing the material on HATYTC, did you search for other teaching material on you tube by yourself?	Yes	63%
	No	27%
If yes, what kind of material did you look for? (choose only the answer that best reflects your opinion)	More Anatomy Videos	49,2%
	You tube channels related to other subjects (e.g. biology, histology, general pathology,embryology)	39,7%
	Other:	11%

the way students study. This result therefore confirms the general data on the digital Native generation also at Italian level on this specific population of students.

The results obtained by the third section therefore confirm what Anderson (2009) said, which underlines how social networks such as Facebook can encourage students to share themes and interests usually excluded from the typical interactions of traditional courses.

Our results of the fourth section therefore shows that HATYTC has functioned as a sort of 'forerunner' for the autonomous search for in-depth material on the web, not only for anatomy, but also for other areas of medicine, thus promoting the acquisition of an autonomous and transferable method of study, a transversal competence and continuous learning.

The analysis of data belonging from fifth section shows that the two media are used for different purposes: HATYTC is appreciated for its effects on learning, while HATPFP for communication and relation with the teacher. This result shows an important distinction to be aware of when designing the didactic uses of technolo-



Graph 4. Student opinion on the usefulness of the two social media in the study of anatomy.

gies; this result warns us that the properties of different media are linked to different functions in the educational process (i.e. social networks such as facebook to communication functions, social media such as youtube to information-conscious functions) and how it is appropriate to think of heterogeneous configurations or ecologies of media rather than a single technology (Heath and Luff, 2000; Suchman, 2007; Alby and Zucchermaglio, 2008).

Limits of the study

Among the limits of the study we can list the following: 1) it is a study that concerns a practice used by a single teacher, it would be interesting to do other studies that also involve other anatomy courses taught by other teachers who use the same educational practice; 2) it is an exploratory study for which survey items could have been analyzed further, for example, taking into account the affiliations of students to different degree courses; 3) the students who answered the questionnaire belong to a specific category of students: this on the one hand places limits on the possibility of generalizing the results, on the other hand gives an account of their ecological validity and applicative utility in terms of feedback for the educational design of the course in question.

Conclusion

The use of HATPFP and HATYTC by teachers and students in the study of anatomy has been positively perceived in several international studies (Cho, Hwang, 2011; Craig et al.2010; Drake et al. 2009 Jaffar 2014, Topping 2014). Our data, the first referring to the Italian context, document that the use of social media Facebook and You Tube in the teaching of Human Anatomy has been perceived by most students as useful and positive. Although within the limits of an exploratory study, we have highlighted how social media can be an effective support for anatomy teaching by facilitating social interactions (in terms of time reduction, simplification, immediacy, less formality), improving learning (in terms of memorization and understanding of concepts: and notions of anatomy), and making students autonomous in their search for new knowledge of anatomy and access to other information resources in other areas of medicine.

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Supplemental material

The Pilot study survey

	Question	Answer
1	Which degree course are you enrolled in?	
2	How old are you?	
3	Please indicate your gender	Male -female
4	Did you already have a Facebook profile before entering university?	Yes/no
	If the answer to question 4 is YES answer questions 5 and 6	
5	Did you use it only for personal contacts (friendships, cultural interests, etc.)?	Yes/no
6	Did you use it for contacts with high school teachers?	Yes/no
7	Did you ever use You Tube before entering university?	Yes/no
8	Did you use it only to display content?	Yes/no
9	Did you use it to view content and upload videos prepared by you?	Yes/no
10	Did you use HATPFP?	Yes/no
11	What did you use it for? List the main uses (open answer)	
12	Do you think HATPFP is a useful way to communicate with the anatomy teacher?	Yes/no
13	Why? (open answer)	
14	Do you consider HATPFP to be a useful way of promoting anatomy learning?	Yes/no
15	Why? (open answer)	
16	Did you use HATYTC?	Yes/no
17	Do you think that HATYTC is a useful tool to promote the learning of anatomy?	Yes/no
18	Why is that? (open answer)	
19	After viewing the material on HATYTC, did you search for other teaching material on you tube by yourself?	Yes/no
20	If yes, which one? (open answer)	
21	Which of HAT social media was most useful in anatomy learning?	HATPFP/ HATYTC/ both/ none

The multiple choice survey

Section		Question	Answer
First	1	Which degree course are you enrolled in?	
	2	How old are you?	
	3	Please indicate your gender	Male -female
Second	4	Did you already have a Facebook profile before entering university?	Yes/no
	5	Did you ever use You Tube before entering university?	Yes/no
Third	6	Did you use HATPFP?	Yes/no
	7	If Yes, What did you use it for? Select all applicable items	To ask the teacher for examination Details To ask the teacher Lesson details (place/ time) To get information about seminars To get information about video published in the HATYTC To acquire materials and files for deepening the lessons To request for explanations on topics dealt with in class
	8	Do you think HATPFP is a useful way to communicate with the anatomy teacher?	Yes/no
	9	If yes, for what communicative characteristic? (select all applicable items)	Fast communication Informality of communication Ease of use
Fourth	10	Did you use HATYTC?	Yes/no
	11	Based on your experience, do you think that HATYTC is a useful tool to support anatomy learning?	Yes/no
	12	If yes, why? (choose only the answer that best reflects your opinion)	Because videos allow to easily visualize the studied concepts Because videos implement textbook content Because videos allow to understand the three-dimensional relationships between organs Because videos facilitates anatomy understanding and memorization Other:
	13	After viewing the material on HATYTC, did you search for other teaching material on you tube by yourself?	Yes/no

	14	If yes, what kind of material did you look for? (choose only the answer that best reflects your opinion)	More Anatomy Videos youtube channels related to other subjects (e.g. biology, histology, general pathology, embryology) Other:
Fifth	15	Which of HAT social media was most useful in anatomy learning?	HATPFP/ HATYTC/ both/ none

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Research Article - Basic and Applied Anatomy

Situs inversus totalis in a 96-year-old female cadaver: evidence pointing toward the two-cilia model

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Abstract

Situs inversus totalis is a complete transposition of the thoracic and abdominal organs and represents one of many laterality defects within the heterotaxy spectrum. Here we report a case of a 96-year-old female cadaver with situs inversus. Examination of the respective computed tomography images revealed situs inversus totalis with dextrocardia. A detailed dissection demonstrated the transposition of the viscera and confirmed complete reversal of the visceral organs without major anomalies. In order to gain insight into the etiology of the present anatomical anomaly, we performed a screen for the putative genetic variants in the coding regions (exomes) of the DNA extracted from the cadaveric tissue using Next Generation Sequencing (NGS) technology. The analysis of the data revealed the presence of genetic variants, *DVL1*, *DNAH9*, *PKD1*, and *TRPV4*, that are closely associated with the regulation of cilia structure and function. Aberrant cilia function has been proposed by other investigators to be a major cause of laterality defects. Because *DNAH9* mutation could be linked to the impairment of motile primary cilia function and mutations in *DVL1*, *PKD1*, and *TRPV4* to that of non-motile primary cilia, our data could provide evidence in support of the two-cilia model where a synergistic functioning of both cilia types is required for a proper activation of the asymmetrical Nodal cascade in the left lateral plate mesoderm thereby assuring a typical and complete asymmetrical patterning of visceral organs.

Keywords

Situs inversus totalis, dextrocardia, computed tomography, DNA exome sequencing, next generation sequencing, two-cilia model.

Introduction

Situs inversus represents a group of congenital disorders characterized by reversal of visceral organs along the left-right axis. It falls within the heterotaxy spectrum which defines the orientation of the visceral organs in the thorax and abdomen and includes situs solitus (normal), situs ambiguous (heterotaxy) and situs inversus totalis (reversed). The latter condition represents a complete mirrored image of situs solitus and has an incidence of 1 in 10,000 (Kim, 2011; Lin et al., 2014). In situs inversus totalis, the apex of the heart is pointed to the right (dextrocardia) with a bilobed right lung and a trilobed left lung in the thoracic cavity. The abdominal viscera are also reversed with the liver being located in the left upper quadrant and the stomach and spleen being located in the right upper quadrant of the abdominal cavity. It is

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well established that the disruption of the left-right axis, also called laterality, causes situs inversus totalis (Nonaka et al., 1998; Vandenberg and Levin, 2013; Blum et al., 2014) and dysfunctional cilia at the embryonic node is a major contributor to laterality defects (Nonaka et al., 1998; Yoshiba and Hamada, 2014; Pennekamp et al., 2015). The organ arrangement has been attributed to improper initiation by the primary and motile cilia of the leftward asymmetric genetic flow in the lateral plate mesoderm during gastrulation (Nonaka et al., 1998; Bisgrove and Yost, 2006; Fliegauf et al., 2007; Leigh et al., 2009; Pennekamp et al., 2015). The left-right asymmetrical distribution of visceral organs is one of the major biological processes underlying the normal development of the human body and gaining more insight into its molecular mechanism(s) would be very important for our better understanding and treatment of the pathologies associated with the incorrect left-right viscera patterning (Burton et al., 2014; Ekbote et al., 2014; Elder et al., 2014).

The purpose of this study is to describe the anatomical characteristics of situs inversus in a 96-year-old female cadaver as well as to identify novel genetic variants associated with the development of situs inversus in humans. To the best of our knowledge, this is the first comprehensive characterization of situs inversus in the human body which includes computed tomography (CT) imaging, gross anatomical examination, and genetic analysis.

Materials and Methods

Human cadaveric body procurement

A 96-year-old female cadaver was received through Saint Louis University (SLU) School of Medicine Gift Body Program from an individual who had given her written informed consent. The body was embalmed through the right femoral artery with a solution (2:1 ratio) of water and mixture containing 33.3% glycerin, 28.8% phenol, 4.6% formaldehyde, and 33.3% methanol. The medical record indicated a presence of situs inversus without further specification.

Computed tomography imaging

The cadaver underwent a CT scanning at SLU Hospital using Siemens SOMATOM Definition Flash system (140 kV; 119 mAs; slice thickness: 3 mm by 3 mm interval; detector size: 0.6 mm, 128 rows of detectors, pitch of 0.6). A total of 83 coronal, 397 axial, and 140 sagittal images were obtained with standard resolution and analyzed using Syngo Fast-View software to determine the type of situs inversus in the cadaveric body. An Interactive Atlas of Human Anatomy (IMAIOS) and Imaging Atlas of Human Anatomy (Weir et al., 2011) were used to assure a correct identification of the major viscera on the CT scans.

Anatomical dissection

Dissection procedures were conducted according to Casey and Campeau (2014) and Delton (2017). To that end, a Y-shaped incision was made to reflect the skin and

expose the underlying muscles, rib cage, parietal pleura, and peritoneum. The incision was made from the right and left clavicles to the midline of the body down to the superior surface of the pubic symphysis. After the skin was reflected, the muscles were removed from the rib cage and the ribs were cut by a Stryker saw along the midaxillary line of the thoracic wall from rib 10 to the clavicle and then to the sternum. The thoracic and abdominal viscera were examined and each dissection step was thoroughly documented using a Canon EOS 350D/ Digital Rebel XT camera.

Thorax

The great vessels of the neck and the right and left phrenic and vagus nerves were dissected before opening the pericardial sac. Once the pericardium was cut, reflected and cleaned, the major vasculature of the heart was exposed. The direction of the apex of the heart and the number of lobes on each lung were documented. The hila of the lungs were cut to remove the lungs from the thoracic cavity. The major vasculature and the bronchial tree were identified in the hilum of each lung. The great vessels of the heart were sectioned to remove the heart from the pericardial sac. Each functional chamber of the heart was cleaned and identified. The structures of the posterior mediastinum were dissected.

Abdomen

After major viscera, vasculature and nerves had been identified in the thoracic cavity, the abdominal structures were then examined. The diaphragm was cut in the midline and reflected to show all the borders of the liver. The portal triad was dissected and then cut midway at the hepatoduodenal ligament. The inferior vena cava and ligaments of the liver were cut to remove the liver and the vasculature and biliary system were then documented. Next, the three unpaired branches (celiac trunk, superior mesenteric artery and inferior mesenteric artery) from the abdominal aorta were cleaned and exposed. The vasculature of the entire digestive tract was cleaned and identified. The small intestines were reflected superiorly in order to view the abdominal aorta and inferior vena cava. Lastly, the kidneys and corresponding vasculature as well as the spleen were studied.

Genetic Analysis

Next Generation Sequencing (NGS) and bioinformatics analysis were performed as previously described (Frolov et al., 2018) with the following modifications. DNA was extracted from lung specimen procured from the embalmed human body and was sequenced to 30x depth of coverage (~4.5 Gb) on the Illumina HiSeq 2500 NGS platform. The 30x depth of coverage fulfills a requirement for the detection of human genome mutations (10x to 30x, Illumina). DNA extraction and exome sequencing were conducted by Omega Bioservices (Norcross, GA). The bioinformatics analysis (the variant calls and annotation of genetic variants) was performed by Genome Technology Access Center (GTAC) at Washington University in St. Louis. Genetic variants with deleterious (pathologic) amino acid substitutions were identified through the consecutive filtering steps described in Table 1. More specifically, *Step 1* allows

Table 1. Filtering steps to identify rare deleterious (pathologic) amino acid substitutions in mutant proteins associated with the present case of situs inversus.

Step 1	Remove synonymous, non-frameshift deletion, non-frameshift insertion, unknown, and not available (NA) variants.
Step 2	Remove variants in the ExAc 65,000 exomes with minor allele frequency (MAF) > 0.01.
Step 3	Keep SIFT-predicted, disease-associated (D) and NA variants.
Step 4	Keep PolyPhen_2-HDIV -predicted D, pathologic (P), and NA variants.
Step 5	Keep PROVEAN-predicted D and NA variants.

a removal of the genetic variants that will not change the encoded amino acid due to the degenerate nature of genetic code (synonymous mutation) or least likely to affect a correct transcription of the respective genes (non-frameshift deletion and non-frameshift insertion mutations) thereby not resulting in aberrant protein structures. *Step 2* is taking out of consideration a bulk of common genetic variants in human population. *Steps 3-5* select proteins with predicted deleterious (pathologic) amino acid substitutions by applying sequentially (to the genetic variants remaining after *Step 2*) a SWIFT (Sim et al. 2012), PolyPhen_2-HDIV (Adzhubei et al., 2010), and PROVEAN (Choi and Chan, 2015) algorithms. The UniProtKB Protein knowledgebase as well as the information extracted from the literature obtained through PubMed and Google Scholar database searches were used for the functional annotation of the resultant genetic variants (mutations).

Results

Anatomical characterization: CT imaging

From the 397 axial images obtained, 4 thoracic vertebral levels (TV2, TV3, TV5, and TV8) and 4 lumbar vertebral levels (LV1, LV2, LV4, and LV5) were chosen to document the major viscera and vasculature of the thorax and abdomen.

At the 2nd thoracic vertebral level (TV2), from anterior to posterior, the right and left brachiocephalic veins were identified deep to the sternum. The brachiocephalic trunk branched from the arch of the aorta on the left, while the right common carotid and right subclavian arteries branched independently on the right. The right and left lungs were identified on each side of the mediastinum with the trachea and esophagus located slightly left of the midline (Fig. 1).

At the 3rd thoracic vertebral level (TV3), the right brachiocephalic vein coursed obliquely to the left before it joined the left brachiocephalic vein to form the superior vena cava. The arch of the aorta was observed and extended from left to right. The trachea was posterior to the aortic arch and anterior to the esophagus (Fig. S1).

At the 5th thoracic vertebral level (TV5), the ascending aorta was posterior to the left side of the sternum and located to the left of the middle thoracic cavity. The azygos vein was located posteriorly and medially to the left-sided superior vena cava. The pulmonary trunk traveled posteriorly and slightly to the right of the arch of the

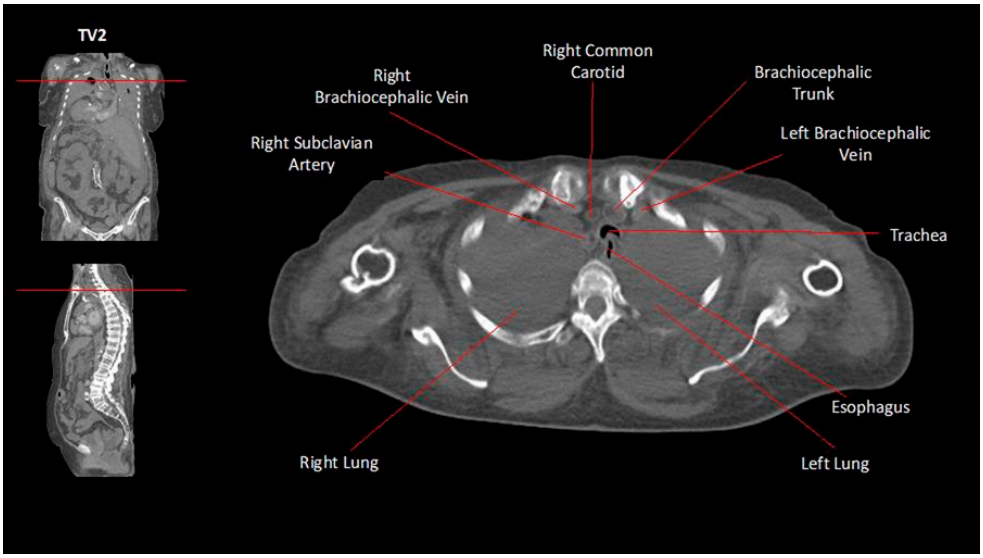

Figure 1. CT imaging of the great vessels of the superior mediastinum at the thoracic vertebral level 2 (TV 2). The right common carotid artery and right subclavian artery are identified as direct braches from the arch of the aorta and the brachiocephalic trunk is identified on the left. The red lines in the coronal and sagittal images denote the vertebral level.

aorta before branching into the left and right pulmonary arteries. The descending (thoracic) aorta was identified to the right of the vertebral column. The esophagus was to the left of the descending aorta (Fig. S2).

The chambers of the heart were identified at the 8th thoracic vertebral level (TV8), and the apex of the heart pointed to the right (dextrocardia). Both functional right atrium and ventricle were posterior to the sternum with the atrium located on the left side. The functional left atrium was anterior to the esophagus and the descending aorta was located to the right of the vertebral column. The functional left ventricle was identified with the outflow tract ending at the aortic valve (Fig. 2).

The major abdominal viscera were identified at the 1st lumbar vertebral level (LV1). On the left side of the abdomen, the liver was identified, while the stomach and spleen were located on the right side. The pancreas traversed the abdomen with the tail of the pancreas pointing to the hilum of the spleen. The left renal vein drained into the inferior vena cava on the left side of the vertebral column (Fig. 3). The right renal vein passed anterior to the abdominal aorta and drained into the inferior vena cava on the left at the 2nd lumbar vertebral level (LV2) (Fig. 4).

At the 4th lumbar vertebral level (LV4), the ascending colon and cecum were identified on the left side, while the descending colon was located on the right side of the abdomen. The right and left common iliac arteries bifurcated from the abdominal aorta (Fig. S3). The common iliac veins ran parallel to the common iliac arteries at the 5th lumbar vertebral level (LV5) before they joined to form the inferior vena cava (Fig. S4).

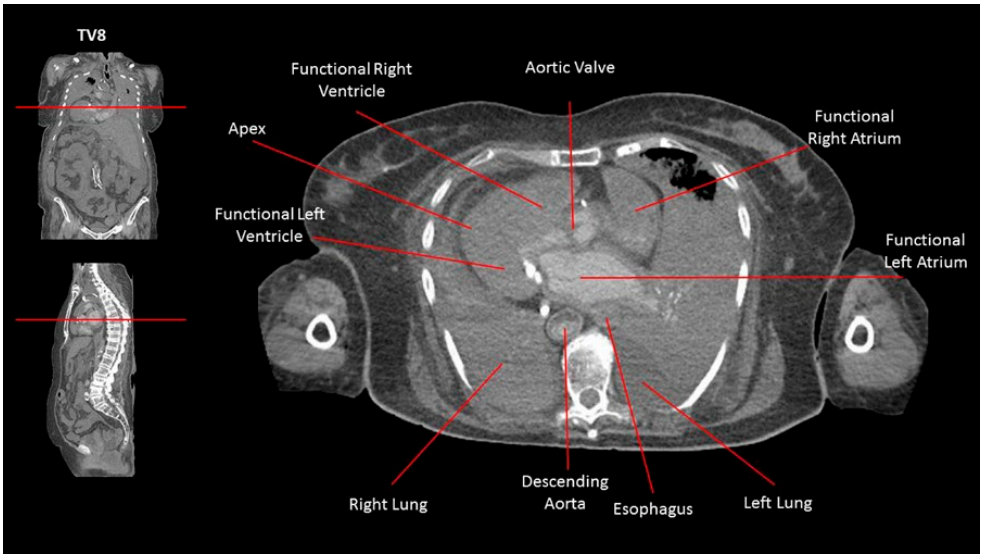

Figure 2. CT imaging of the heart chambers at the thoracic vertebral level 8 (TV 8). The functional right atrium and functional right ventricle are the most anterior chambers located posterior to the sternum. The functional left atrium is located just anterior to the descending aorta and esophagus. The functional left ventricle with its apex of the heart is directed towards the right. The descending aorta can be seen to the right of the vertebral column while the esophagus is located just left of the midline. The red lines in the coronal and sagittal images denote the vertebral level.

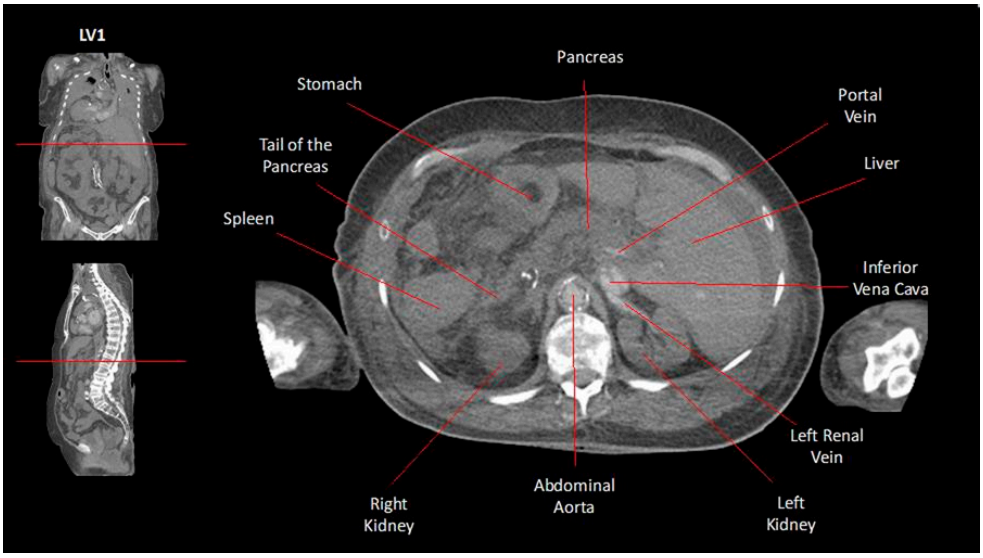

Figure 3. CT imaging of the abdominal viscera at the lumbar vertebral level 1 (LV1). The liver is located on the left side of the abdomen while the stomach and spleen are located on the right. The tail of the pancreas is toward the right. The inferior vena cava is to the left of the abdominal aorta and posterior to the liver. The red lines in the coronal and sagittal images denote the vertebral level.

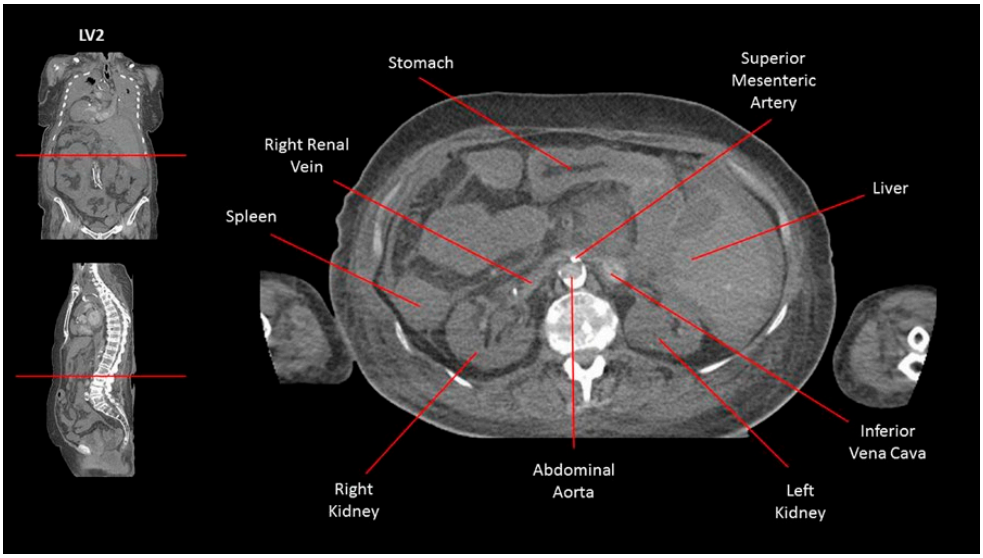

Figure 4. CT imaging of the right renal vein at the lumbar vertebral level 2 (LV2). The right renal vein passes anterior to the abdominal aorta and posterior to the superior mesenteric artery before draining into the inferior vena cava on the left side of the abdomen. The red lines in the coronal and sagittal images denote the vertebral level**.**

Anatomical characterization: Gross dissection

In the thoracic cavity, the cadaver presented with dextrocardia (apex of the heart pointing to the right) with a bilobed right lung and a trilobed left lung (Fig. 5). The right brachiocephalic vein traveled obliquely from the right side of the superior thorax to the left to join with the left brachiocephalic vein to form the left-sided superior vena cava. The superior vena cava then emptied into the functional right atrium. The aortic arch began anteriorly and ended posteriorly at the sternal angle level. The brachiocephalic trunk, right common carotid artery, and right subclavian artery branched from the arch of the aorta from left to right, respectively. The right and left phrenic nerves passed anterior to the hila of the lungs, and the left and right vagus nerves passed posterior to the hila of the lungs (Fig. S5).

The major vasculature of the heart was identified. The direct branches from the ascending aorta, the right and left coronary arteries, were identified in the coronary sulcus. From the anterior view of the heart, the left coronary artery gave off the left marginal artery at the inferior border of the heart. The anterior interventricular artery, a branch of the right coronary artery, was identified in the anterior interventricular sulcus traveling with the great cardiac vein towards the apex of the heart. From the posterior view of the heart, the continuation of the left coronary artery was viewed as it gave off the posterior interventricular branch which traveled with the middle cardiac vein in the posterior interventricular sulcus. The right circumflex artery wrapped around the right side of the heart in the coronary sulcus with the small cardiac vein

and ended before reaching the posterior aspect of the heart. The great, middle and small cardiac veins finally drained into the coronary sinus (Fig. S6).

The chambers of the heart were identified and were named based on function, not anatomical position. The chambers of the left side of the heart (left atrium and left ventricle) and the right side of the heart (right atrium and right ventricle) were named functional right atrium, functional right ventricle, functional left atrium, and functional left ventricle, respectively. Each functional chamber was found to have the characteristic structures typical of situs solitus (Fig. S7). The functional right atrium received venous blood from the superior vena cava, inferior vena cava, and coronary sinus. Within the chamber, pectinate muscles fanned out from the crista terminalis. The fossa ovalis was identified in the interatrial septum. Between the functional right atrium and functional right ventricle there was the tricuspid valve. Within the functional right ventricle, the anterior, posterior, and septal papillary muscles were identified with chordae tendineae connecting them to the cusps of the tricuspid valve. Trabeculae carneae were identified on the wall of the ventricle. The septum trabecula (moderator band) was identified, forming a bridge between the interventricular wall and the anterior papillary muscle. The conus arteriosus led into the pulmonary trunk toward the right. The functional left atrium received two pairs of the pulmonary veins from each side. The cusps of the mitral valve were observed between the functional left atrium and functional left ventricle. Finally, within the functional left ventricle, the anterior and posterior papillary muscles were connected to the cusps of the mitral valve by chordae tendineae. Trabeculae carneae were also identified on the wall of the functional left ventricle. The aortic vestibule ended at the aortic valve (Fig. S7).

Once the lungs were removed from the thoracic cavity, each hilum was examined. The right lung presented with two lobes, superior and inferior, separated by the oblique fissure. The lingula and the cardiac notch were located on the anterior border of the inferior part of the superior lobe. At the hilum of the right lung, the right pulmonary artery was superior, the primary bronchus was posterior, and the superior and inferior pulmonary veins were inferior. The left lung presented with three lobes. The oblique fissure separated the superior and middle lobes from the inferior lobe and the horizontal fissure separated the superior lobe from the middle lobe. At the hilum of the left lung, the left pulmonary artery, the primary bronchus, and the superior and inferior pulmonary veins had a similar arrangement to that of the right lung (Fig. S8).

In the posterior mediastinum, the left and right vagus nerves passed posterior to the hilum of the lungs and approached the esophagus where they contributed to the esophageal plexus. Towards the caudal end of the esophagus, the esophageal plexus narrowed and continued as the anterior and posterior vagal trunks where the right vagus nerve contributed to the anterior vagal trunk and the left vagus nerve contributed to the posterior vagal trunk. The azygos vein emptied into the superior vena cava on the left side of the thoracic cavity over the hilum of the right lung. The hemiazygos and accessory hemiazygos veins were found on the right side and both passed anterior to the vertebral bodies to the left side of the thorax, posterior to the esophagus at the TV9 level and TV8 level, respectively. The thoracic duct was identified between the descending aorta and azygos vein, and crossed to the right side at the level of TV5 and drained into the junction between the right jugular vein and right subclavian vein at the root of the neck (Fig. S9).

In the abdominal cavity, the liver was located in the left upper quadrant of the abdomen. The esophagus passed through the diaphragm at the TV10 level and ended at the cardiac notch of the stomach on the right side of the abdominal cavity (Fig. 5). The 1st part of the duodenum began its descent on the left side of the abdomen at the LV1 level, and crossed transversely to the opposite side at the LV3 level (Fig. 6). The jejunum primarily occupied the right upper quadrant and the ileum was mostly identified in the left lower quadrant of the abdomen with no sign of malrotation. The cecum was identified in the left lower quadrant, and it connected to the terminal ilium and continued as the ascending colon. The transverse colon began at the left colic flexure and traveled horizontally across to the right colic flexure where it became the descending colon. The sigmoid colon continued from the descending colon and traveled into pelvic cavity on the right side and ended at the rectum. A single spleen was located in the right upper quadrant inferior to the diaphragm.

The major paired/unpaired branches from the abdominal aorta were identified. The celiac trunk usually branches into three arteries, but in this study the celiac trunk bifurcated into the splenic artery and the right gastric artery (Fig. S10). The common hepatic artery shared a common trunk with the superior mesenteric artery. The right gastric artery traveled through the hepatogastric ligament to supply the lesser curvature of the stomach. The splenic artery traveled toward the spleen on the right side (Fig. S11). The common hepatic artery ran toward the left and gave off the inferior pancreaticoduodenal artery, gastroduodenal artery, and continued as the proper hepatic artery. The superior pancreaticoduodenal arteries branched from the gastroduodenal artery to provide blood supply to the head of the pancreas and duodenum. In this study, the inferior pancreaticoduodenal artery branched from the common hepatic artery to supply the same area as the superior pancreaticoduodenal artery (Fig. S12).

The portal triad, consisting of the portal vein, proper hepatic artery with an accessary hepatic artery in this case, and common bile duct, was identified in the hepatoduodenal ligament. The common bile duct joined with the major pancreatic duct and opened into the major papilla of the duodenum on the left side of the abdomen. The portal vein was formed by the splenic vein and the superior mesenteric vein dorsal to the neck of the pancreas. The proper hepatic artery gave off left and right hepatic arteries and an additional common trunk for an accessory hepatic artery and left gastric artery (Figs. S13 & S14). The right hepatic artery and accessory hepatic artery accompanied each other to the right functional lobe of the liver.

The branches of the superior mesenteric artery were also identified (Fig. S15). The intestinal arteries passed in the mesentery proper to supply the jejunum and ileum. The middle colic artery traveled through the transverse mesocolon to provide blood supply to the left two-thirds of the transverse colon. The left colic artery traveled retroperitoneally to provide blood supply to the ascending colon. Lastly, the ileocolic artery traveled inferiorly and laterally through the mesentery proper to the terminal ileum, cecum, and lower ascending colon on the left side in the abdominal cavity.

The branches from the inferior mesenteric artery were examined (Fig. S16). The right colic artery traveled retroperitoneally to supply the descending colon on the right side. The sigmoid branches traveled through the sigmoid mesocolon to supply the sigmoid colon. The inferior mesenteric artery continued as the superior rectal artery in the pelvic cavity to supply the superior portion of the rectum. Branches

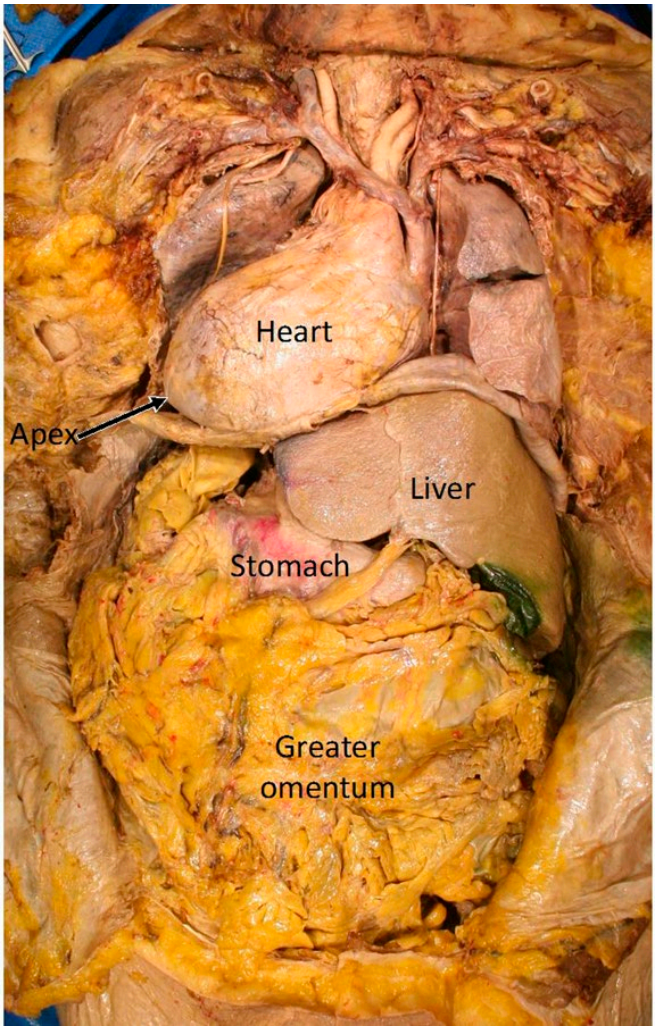

Figure 5. Gross dissection to examine the internal viscera of situs inversus totalis. The apex of the heart is directed to the right (dextrocardia) in the thoracic cavity. The liver is located in the left upper quadrant, and the major part of the stomach is in the right upper quadrant of the abdominal cavity.

of both mesenteric arteries anastomosed to form the marginal artery of Drummond which supplied the large intestines.

In the posterior part of the abdominal cavity, the inferior vena cava was located on the left side of the abdominal aorta. The inferior vena cava traveled superiorly and posteriorly to the liver where the left, middle, and right hepatic veins drained into it. It then passed through the diaphragm at the TV8 level into the thoracic cavity and drained into the left-sided functional right atrium. The kidneys were named by their anatomical position. The right renal artery branched into the apical, anterior

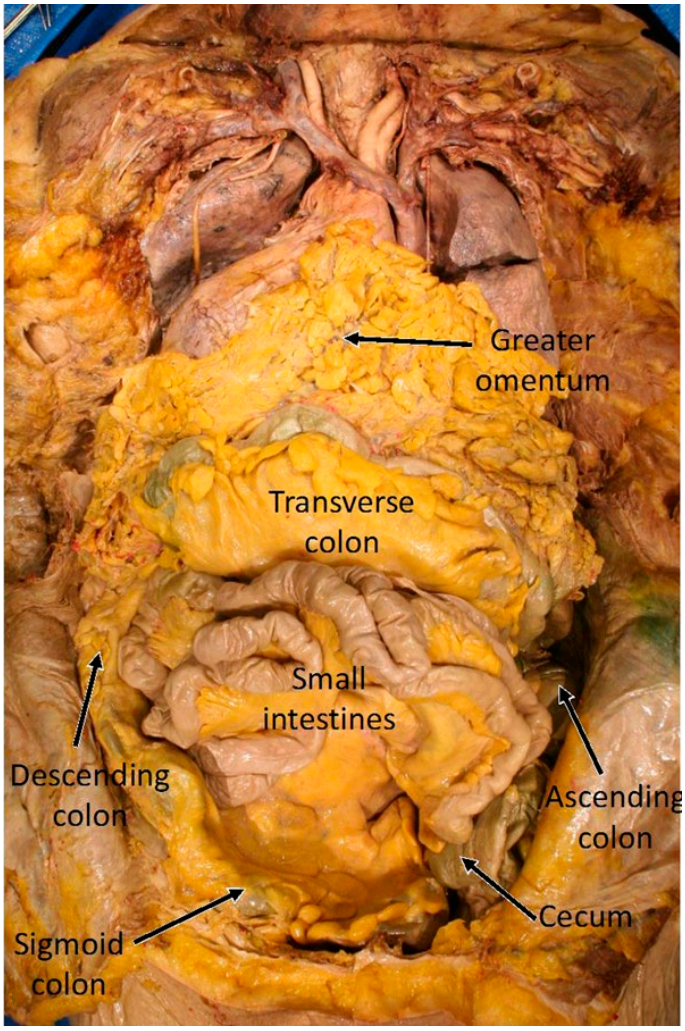

Figure 6. Gross dissection to examine the small and large intestines. The cecum and ascending colon are on the left side, while the descending colon and sigmoid colon are on the right side of the abdomen. The transverse colon travels horizontally from left to right across the abdomen.

superior, anterior inferior, inferior, and posterior renal arteries. Only four branches from the left renal artery were observed and they were the apical, anterior superior, anterior inferior and posterior renal arteries. In this study, the left inferior renal artery branched directly from the abdominal aorta. The right renal vein received a common trunk of the right inferior phrenic vein and suprarenal vein superiorly and the ovarian vein inferiorly, and then passed anterior to the abdominal aorta and posterior to the superior mesenteric artery to drain into the inferior vena cava. The left renal vein drained directly into the left-sided inferior vena cava. Both ovarian arter-

ies branched directly from the abdominal aorta, while the right ovarian vein drained into the right renal vein and the left ovarian vein drained directly into the inferior vena cava (Figs. S17 & S18). Summary of the gross anatomical dissection is presented in the supplementary Tables S1 & S2. Altogether, the results of both the CT imaging and gross anatomical dissection characterize the present case as situs inversus totalis with dextrocardia.

Genetic analysis

The cumulative exome coverage at ≥ 10X depth of coverage was limited to 40.2% due to DNA degradation. In total 19,303 genetic variants were detected. The variants with predicted deleterious (pathological) substitution of amino acids in the mutant proteins were selected through sequential stringent filtering steps that included analysis provided by well established SWIFT (Sim et al., 2012), PolyPhen_2-HDIV (Adzhubei et al., 2010), and PROVEAN (Choi and Chan, 2015) algorithms and servers (Table 1). From the 134 genetic variants predicted to be pathologic (Table S3), seven, *BBS1*, *C5orf42*, *DYNC2H1*, *DVL1*, *DNAH9*, *PKD1* and *TRPV4* could have a negative effect on cilia structure and function thereby leading to the development of situs inversus (Nonaka et al., 1998; Bisgrove and Yost, 2006; Fliegauf et al., 2007; Leigh et al., 2009; Pennekamp et al., 2015). However, the first three genetic variants, *BBS1*, *C5orf42*, and *DYNC2H1,* have been previously linked to several well described human genetic disorders including, respectively, Bardet-Biedl syndrome (Ajmal et al., 2013; Ece Solmaz et al., 2015), oral-facial-digital syndrome VI (Karp et al., 2012), and short-rib polydactyly syndrome (Mei et al., 2015; Okamoto et al., 2015). Because none of the pathologies related to the above syndromes have been identified in the current study, the remaining four variants, *DVL1*, *DNAH9*, *PKD1,* and *TRPV4,* are the ones most likely associated with the development of situs inversus totalis, primarily through the impairment of cilia structure and function (Table 2). All four of these

Table 2. Deleterious (pathologic) genetic variants associated with the current case of situs inversus totalis, that regulate cilia structure and function.

Gene	Protein Function	Variant
DVL1	Dishevelled segment polarity protein 1. Plays a role both in canonical and non-canonical Wnt signaling. Participates in Wnt signaling by binding to the cytoplasmic C-terminus of frizzled family members and transducing the Wnt signal to down-stream effectors.	p.Arg108Trp
DNAH9	Dynein heavy chain 9, axonemal. Force generating protein of respiratory cilia. Produces force towards the minus ends of microtubules.	p.Arg3447His
PKD1	Polycystin-1. Acts as a regulator of cilium length, together with PKD2. The dynamic control of cilium length is essential in the regulation of mechanotransductive signaling.	p.Arg3620Trp p.Arg3621Trp
TRPV4	Transient receptor potential cation channel subfamily V member 4. Non-selective calcium permeant cation channel involved in osmotic and mechanosensitivity. Primary cilia receptor.	p.Leu223Val p.Leu257Val

variants are novel as they have never been linked before to the laterality disorders in humans including situs inversus totalis (Deng et al., 2015).

Discussion

The case of a 96-year old female cadaver presented in the current study can be described as situs inversus totalis with dextrocardia. The latter is a rare condition only seen in 0.01% of the population. Individuals with situs inversus totalis usually have a normal life expectancy and the risk of contracting a disease is similar to that of patients with situs solitus (Kumar et al. 2014). Our description of the present case as situs inversus totalis was based on the completely reversed orientation of the thoracoabdominal viscera which was unequivocally supported by the respective CT imaging data and the anatomical dissection results. In addition, we noted a presence of various minor vascular anomalies within the abdomen that are described below.

In anatomically typical individuals, the celiac trunk usually branches as a triad of three arteries: left gastric artery, splenic artery and common hepatic artery. In the present case, the celiac trunk bifurcated into the splenic artery and left gastric artery. Yet the common hepatic artery and superior mesenteric artery shared a common trunk, and the left gastric artery and an accessory hepatic artery shared a common trunk that branched from the proper hepatic artery. The accessory hepatic artery passed through the hepatoduodenal ligament and traveled with the right hepatic artery to the functional right lobe of the liver. The inferior pancreaticoduodenal artery branched from the common hepatic artery instead of the superior mesenteric artery. Finally, the left inferior renal artery branched directly from the abdominal aorta.

It is well established that cardiac anomalies are common in patients with situs ambiguous, while 90-95% of patients with situs inversus totalis have a typical internal cardiovascular structure (Applegate et al., 1999; Hur et al., 2014), which was observed also in the current study. However, even though the heart structures were reversed as compared to normal anatomy, the morphology of each heart chamber was unremarkable. In addition, the formation of inferior vena cava and azygos vein were also reversed but normal which is unlike other cases of heterotaxy, where the azygos vein being the continuation of the inferior vena cava represents the most common cardiovascular anomaly (Applegate et al., 1999; Maldjian and Saric, 2007; Kumar et al., 2014; Ghorbnazadeh et al., 2017).

In order to gain insights into the etiology of the current case, we performed a screen for the putative genetic variants associated with situs inversus totalis by employing NGS of the DNA extracted from the body used in the current study. The respective bioinformatics analysis identified four most relevant variants, *DNAH9*, *PKD1*, *TRPV4*, and *DVL1*, all of them being involved in the regulation of cilia structure and function. *DNAH9* is a human homolog of mouse *Dnahc9* which encodes a dynein heavy chain 9, an important axonemal component of cilia, and its mutation could lead to the impairment of motile cilia (Thomas et al., 2010; Huang et al., 2015). Earlier studies showed that defects in motile cilia could result in the randomization of the left-right axis during early embryogenesis (Nonaka et al., 1998; Tabin and Vogan, 2003) thereby promoting the development of laterality defects. On the other hand, *PKD1*, *TRPV4*, and *DVL1* genetic variants could be responsible for the impair-

ment of primary cilia function. Studies on *Pkd1*, also known as polycystin-1, demonstrated its close involvement in the mechanosensation coupled with the intracellular calcium signaling in the primary kidney cilia where it co-distributes and functions in the same mechanotransduction pathway with polycystin-2 (*Pkd2*) (Nauli et al., 2003). More importantly, it has been also reported that *Pkd2* deficient mice displayed a complex spectrum of laterality defects (Yoshiba et al. 2012). *TRPV4* encodes a human homolog of mouse *Trpv4*, a well-characterized cation channel known to be involved in mechanosensation by serving as one of the receptors of primary cilia (Thomas et al., 2010; Lee et al., 2015). It is also well-established that *DVL1* regulates Wnt signaling which utilizes primary cilia as a signaling platform (Simons et al., 2005; Lancaster et al., 2011; McMurray et al., 2013; Oh and Katsanis, 2013).

In summary, the results of our genetic analysis could provide evidence in support of the two-cilia model where both motile primary and non-motile primary cilia work synergistically at the node to initiate the asymmetrical Nodal cascade in the left lateral plate mesoderm (McGrath et al., 2003; Nauli et al., 2003; Yoshiba and Hamada, 2014; Duncan and Khokha, 2016). However, it appears that the disruption of both the motile primary and non motile primary cilia could be a prerequisite for the development of situs inversus totalis described in the present case. In the latter, the disruption of motile primary cilia could be linked to *DNAH9* mutation and the impairment of non-motile primary cilia may result from the mutations in *PKD1*, *TRPV4*, and *DVL1*.

In conclusion, it should be noted that the genetic analysis of human pathologies or anatomical aberrations can only reveal an association of a specific genetic variation(s) with the observed phenotypical changes. A causative link between the particular genotypic and phenotypic changes can only be established by reproducing the latter through either the respective genetic manipulations in the model organisms (systems) or by correcting an existing pathology by targeting a transcription of a mutated gene(s) or the respective protein function. Nevertheless, the associative (in general) nature of genetic analysis, including that presented in the current report, could provide invaluable leads in unraveling new molecular mechanisms governing the development of a human organism, including asymmetrical patterning of visceral organs.

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No competing interest have to be declared.

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Additional information

Supplementary materials are available through: https://drive.google.com/file/d/1x2LXQeRrlyCIxmElO_U7mQdHPfDzyxhv/view

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