IJAE
Vol. 124, n. 3: 247-270, 2019
ITALIAN JOURNAL OF ANATOMY AND EMBRYOLOGY

Discovery and development of the cardiovascular system with a focus on angiogenesis: a historical overview

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Abstract

In comparison with other organs, the beating heart and the red color of blood flowing inside vessels not only were arguments for anatomical research but also inspired metaphorical as well as symbolical considerations. Indeed, for a long time the cardiac pump was thought as the seat of passions and a haemocardiocentric theory developed, especially in Aristotle's philosophy. After the Galen's medicine, new anatomical observations were shown in the Renaissance period, with modern descriptions of the cardiovascular system by Leonardo da Vinci and Vesalius. Descartes' mechanistic view confirmed the discovery of the blood circulation by Harvey, and microscopic investigations unrevealed the capillary network. Most of these studies mainly described blood vessels as static anatomical structures and said little about their formation and development. Then, at the end of the 18th century, Hunter introduced the concept of angiogenesis in *in vivo* experiments, leading to the modern embryological research. The beginning of angiogenesis era was characterized by the first microscopical evidences of capillary formation and the discovery of the angioblasts by Sabin. The evolution of angiogenesis concept occurred in the '70s of 20th century with the pivotal work by Folkman and the onset of research on pro-and anti-angiogenic factors which characterized the next two decades of angiogenesis field. New models of neovascularization have been recently proposed such as the vasculogenic mimicry and the vessel cooption to explain the non-angiogenic tumor growth and the antiangiogenic drug unresponsiveness. Future trends are dealing with the role of angiogenic process and immunity.

Keywords

Angiogenesis, blood vessels, heart, history of medicine, embryogenesis, vasculogenesis, proangiogenic factors, antiangiogenic factors, vasculogenic mimicry, vessel cooption.

Introduction

At the dawn of great civilizations men's curiosity and technology were addressed to the discovery of the nature and its phenomena, and philosophy appealed human conscience with fundamental questions about the meaning of life. Then, living beings were not an exception in this exploration. In particular, the human body gained the interest of scientists and thinkers, because of its special debatable nature: simple material object or the seat where human and divine meet? Anatomical dissection was

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http://www.fupress.com/ijae
DOI: DOI: 10.13128/ijae-11656

performed not only to know structure and organization of the human body, but also to ascertain the secrets of its spiritual nature. Among the different systems explored, the cardiovascular one was the most intriguing and fascinating. Indeed, its holistic organization, and dynamic and functional features, forcefully evoked long-lasting extraordinary theories. The concept of blood "circulation" also needed a long time to be correctly assessed. If anatomy and physiology of the cardiovascular system advanced more or less at the same time, the concept of vessel formation, as well as other anatomical structures, was more difficult to be understood, and it was mainly developed in the embryological field. After the first modern descriptions of foetal blood vessels, dating back to the Renaissance period, it was necessary to wait for the embryological studies of the 19th century to obtain the fundamental concept of angiogenesis. Since then, a large body of evidence was provided, and the modern molecular biology also allowed to discover many factors and mechanisms involved in blood vessel formation, especially in an attempt to modulate this process in cancer disease.

The cardiovascular system: concepts and theories before the 20th century

Ancient times: the cardiovascular system between science and philosophy

Since ancient times, the beating heart and the red blood flowing inside an intricate network of vessels drew men's attention and fantasy. The position of the heart in the centre of the body also contributed to assign a crucial importance for this organ. In particular, perceptible palpitations due to efforts and emotions evoked extraordinary roles for such a vital organ, whose inactivity just coincides with death. For these reasons, the cardiovascular system not only was considered in an anatomical and medical context, but was also endowed with metaphorical as well as symbolical, anthropological, mythological, magical and religious significance. Indeed, for a long time the cardiac pump was thought as the seat of passions, intelligence and soul.

In prehistoric times, about 15,000 years ago, the Paleolithic man realized a stylized heart of mammoth and bison on the wall of the Pindal cave in Spain, and Niaux cave in France. This suggests that the abstract thinking of our ancestors understood the importance of the heart for life when killing animals during hunt. In ancient Mesopotamic civilizations the heart was also recognized as the seat of the soul or spirit, the will and conscience. A very special consideration was elaborated by Egyptian civilization, when the heart was also symbolized into two hieroglyphic signs and was the only organ to be left inside the mummies or replaced with a scarab beetle amulet reporting a dedicated inscription. Again, the judgment of the dead was the process including the famous and typical scene of psychostasia or weighing of souls, when the weight of the heart was compared with that of a feather. In the famous Smith and Ebers papyri, the pulse was directly correlated to the heart activity. Interestingly, the latter papyrus also reported the description of a type of "vessel-tumor", related to the wound of a blood vessel (Latronico, 1955; Marinković et al., 2014; Natale et al., 2017).

Ancient Greek philosophers and scientists gave a great contribute to the development of technology and medicine. In the Homeric era two types of soul were distinguished. One type was named *psyche*, a sort of impalpable breathing entity which was associated to any particular part of the body. It was silent during active life. The

other type was tripartite into different body souls, named *thymos*, *noos* and *menos*, and active during waking life. All were differently located into the chest and in the proximity of the heart. Concerning the identification of a pivotal organ within the human body, two main theories took place. More correctly, in the 5th century BC an encephalocentric theory arose in the pre-socratic era. In the embryology of Anaxagoras the brain was recognized as the first living organ. Again, according to Alcmaeon of Croton, from the school of Pythagoras, as well as to Hippon of Samos, Diogenes of Apollonia and Philolaus of Croton, the brain was considered the most important organ, the mental activity depending on its function. Later, the eclectic physician Hippocrates of Kos (c. 460-370 BC) confirmed the importance of the brain. He described the tumor growth associated with solid columns of infiltrating cancer cells and swollen blood vessels tortuously arranged around the tumor itself, reminiscing the claws of a crab. This impressive iconography of newly formed pathological vessels was particularly referred to breast cancer, and Hippocrates named the tumor *karkinoma* (the Greek name for crab). In the meantime, the famous philosopher Plato (c. 427-347 BC) considered the incorporeal and immortal soul as tripartite: irascible, located in the chest, with the heart as a guardian; concupiscible, located in the abdomen, near the liver; and rational, located in brain, the head having a hierarchical supremacy over the other parts of the body. However, by the Greeks a hemocardiocentric theory was also proposed, stimulated by the suggestive and rooted notion that the heart activity just coincides with life. In the opinion of Empedocles of Agrigento (5th century BC), thoughts originated in the chest, in particular in the blood circulating around the heart, which was considered the centre of *pneuma* (breath and soul). The Empedocles' theory had a great fortune and diffusion and was later incorporated into the authoritative doctrine of the famous Aristotle (384-322 BC). According to the philosopher from Stagira, the brain was described as cold and bloodless, insensitive to touch, unconnected with the sense organs. The soul was the form of the body and the mind did not need an anatomical basis to perform its activity. Apart from the intellectual soul (*nous*), which was immaterial and belonged only to humans, the other soul faculties resided in the heart, considered the seat of life principle, movement and sensation. On the contrary, the brain had the mere function to temper the warmth emanating from the heart, the source of the innate, vital heat. Veins, arteries, and nerves were not distinguished and they were mere paths from the heart to the sense organs and muscles. Aristotle also noted that a chicken decapitated can live and walk for several minutes or more, but with a heart lesion the death is immediate. Furthermore, alterations of the state of mind associates with changes of cardiac rhythm, then the heart was assumed more important and, unlike Plato, considered the supreme organ (Manzoni, 1998; Crivellato and Ribatti, 2007; Santoro et al., 2009; Natale et al., 2017).

Aristotle was also a scientist and he dedicated a particular attention to the description of the natural world. He classified several animal species and investigated natural phenomena in living beings. In this respect, he is generally considered a pioneer in biological studies. His interests mainly dealt with animal locomotion, respiration, aging and sensations. Within a vitalistic epigenetic vision, his embryological studies deserve a particular mention, and some interesting observations allowed Aristotle to be credited with the first description of blood vessel formation. The philosopher noticed that the cardiovascular system is the first structure to appear during chick development. In his *Historia animalium* (History of Animals) he clearly stated

that *the first signs of the embryo are seen after three days and three nights… and the heart is no bigger than just a small blood spot in the white. This spot beats and moves as though it were alive*. More in depth, he understood that blood vessel formation and distribution were related to heart development and function, with the blood flowing inside this system to nourish the whole organism. Then, body growth just depended on the ongoing development and organization of blood vessels and the heart was the first organ to appear during organogenesis and the centre of that system (Crivellato and Ribatti, 2006; Crivellato et al., 2007; Ribatti et al., 2015).

The physician Diocles of Carystus (4th century BC) reinforced the central position and importance of the heart, as the seat for hearing and understanding. He described the auricles, improving the anatomy of this organ, and attributed to these appendages the role of sensory organs. However, he also recognized the involvement of the brain in cognitive functions. In particular, the right half of the brain was the centre for sensation and the left for intelligence, thus anticipating the modern concept of cerebral dominance. These ideas were also accepted by Praxagoras of Cos (born c. 340 BC) and his disciples. This physician had the great merit to distinguish arteries from veins. Nevertheless, only veins were thought to transport the blood, whereas arteries were involved in the distribution of the *pneuma* (Crivellato and Ribatti, 2007).

Later, during the Hellenistic period (3rd century BC), the Alexandrian medical school distinguished itself for a remarkable progress, especially in anatomy, and two principal figures emerged: Herophilus of Chalcedon (335-280 BC) and Erasistratus of Ceos (310-250 BC). These scientists had the opportunity to dissect corpses and vivisect animals and probably human bodies from criminals. This activity led to improve not only anatomy, but also physiology, with the description of the arterial pulse depending on the heartbeat. Finally, the neuroanatomical studies allowed to assign cognitive functions to the brain (encephalocentric theory) to the detriment of the heart, and blood vessels were distinguished from nerves. However, against the evidence of the facts, arteries were still believed to transport a vital spirit. Erasistratus also described a peculiar structure characterized by a network of veins, arteries and nerves, named *triplokia*. (Latronico, 1955; Crivellato and Ribatti, 2007; Natale et al., 2017).

During the Roman period, the ancient knowledge was elaborated by one the most important figures of the western medicine: Galen of Pergamon (2nd century CE) (Fig. 1). His observations and descriptions were dogmatically accepted for about fifteen centuries. One of his most famous physiological theories just concerned the cardiovascular system, which was not regarded as a circulatory one. Apart from the heart, Galen's theory also assigned a pivotal role to the liver, responsible for blood formation and movement (Azizi et al; 2008; Limet, 2010; Aird, 2011). However, in the Middle Ages the influence of Byzantine and Islamic medicine, and the development of the *Schola Medica Salernitana*, favored a new approach. Even if without a practical repercussion, Mondino de Luzzi (c. 1270-1326) dared to propose the dissection of human corpses.

New insights into the anatomy of the cardiovascular system were provided by Islamic medicine. In *The Canon of Medicine* the famous Avicenna (980-1037) admitted the connection between arteries and veins, and a peripheral network of very small vessels was postulated: *The good blood ascends into the superior vena cava, and its subsequent course is into smaller and smaller veins: and finally into the finest hair-like channels ...*

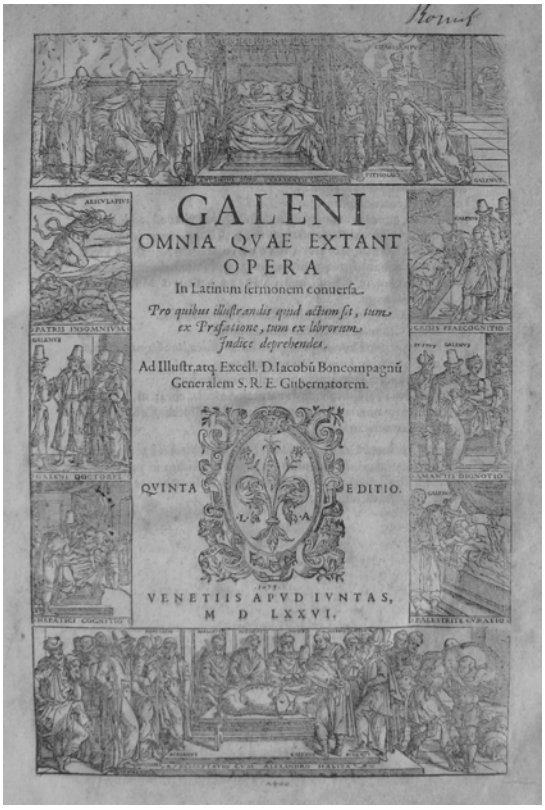

Figure 1. Frontispiece of Galen's *Omnia quae extant opera* (1576). Library of Medicine and Surgery, and Pharmacy. University of Pisa.

according to the decree of Allah. Ibn al-Quff (1233-1286) also hypothesized the presence of communicating fenestrations between arterial and venous systems: *These fenestrations are hidden from the eye*. Ibn al-Nafis (c. 1210-1288) was the first to describe the pulmonary circulation (Azizi et al; 2008; Limet, 2010; Aird, 2011; Natale et al., 2017).

Renaissance of the cardiovascular system

The first scientific and accurate anatomical drawings of the heart and blood vessels were realized by Leonardo da Vinci (1452-1519) in the 15th century. This year we just celebrate the achievements of Leonardo, one of the greatest polymath of all times, who died five-hundred years ago. He revolutionized Renaissance painting, taught and technology, inspiring many important artists and scientists, and his innovative dissections on human corpses anticipated the anatomical revolution by Vesalius. Leonardo invented the anatomical iconography, with remarkable drawings and informative descriptions. He also adopted descriptive models taken from other fields. Then, the anatomical description was inspired by Ptolemy's geography and Leonardo

considered the human body as a new territory to be explored and named: *Thus, in 15 entire figures, you will have set before you the cosmography of this lesser world on the same plan as, before me, was adopted by Ptolemy in his cosmography; and so I will afterwards divide them into limbs as he divided the whole world into provinces*. In particular, blood vessels suggested such an approach: *Here shall be represented the tree of the vessels generally, as Ptolemy did with the universe in his Cosmography; then shall be represented the vessels of each member separately from different aspects*. Leonardo was fascinated by the heart and its valvular functions, and about one fourth of his anatomical drawings, mainly from the Windsor manuscripts, was dedicated to the cardiovascular system. His astonishing embryological drawings of fetuses and newborns are also very famous. Then, after Aristotle, Leonardo also faced the dynamic changes occurring during development, including blood vessel rearrangement. He thought that the fetus had no cardiopulmonary activity: *To this child the heart does not beat, and it does not breathe because it lies continually in water*. Leonardo described the presence and formation of the intricate vascular network interconnecting the fetus with his mother: *The «vene massime» of the child in the uterus. Explain how the veins of the uterus ramify in the uterus, and which and how many they are, and which enter the placenta, and which of them are torn asunder in the separation of the child from the uterus. The veins and arteries of the uterus of woman have such a mixture of contacts with the extreme veins of the navelstring of her child […], as the «vene miseraice» ramifying in the liver, have with the ramification of the veins which descend from the heart into the same liver, and as the ramifications of the veins of the lung have with the ramifications of the trachea, which refresh them. But the veins of the child do no ramify in the substance of the uterus of its mother, but in the placenta, which takes the place of a shirt in the interior of the uterus, which it coats, and is connected (but not united) to this by means of the cotyledons etc*. More interestingly, he also provided the description of a blood vessel that collapses when its function after birth ends. It is the case of the fetal umbilical vein, whose remnant is represented by the round ligament of the liver: *When the umbilical vein is in operation, for which it was created, it attains the principal site in Man, that is, the middle of the body, as well for height as for breadth. But when such vein was afterwards deprived of its office, it drew itself apart together with the liver, created and then nourished by it. And this upper part of the umbilical vein was pushed through the change of the middle of the liver, which through the increasing of the milt, created on the left side [this liver] was driven into the right side and carried with it the upper part of the umbilical vein, which was joined to it* (Vangensten et al., 1911-1916; Aird, 2011; Shoja et al., 2013; Loukas et al., 2016; Kemp, 2019; Laurenza, 2019).

Leonardo noticed different patterns of vessel ramification and in the pre-microscopic era intuitively perceived the existence of capillaries: *Of the ramifications of the veins are two sorts, i.e. simple and compound ones; simple is the one which goes on ramifying infinitely; compound is it, if from the two ramifications a single vein is generated, as you see* n m *and* m o, *branches of two veins which join in* m *and compose the vein* m p, *which goes to the membrum* (Fig. 2). More in depth, he clearly defined these narrower blood vessels: *…per le istrette ramificazion delle vene capillare* (through the narrow ramifications of capillary veins). Leonardo also dealt with blood vessel ramification and distribution: *Always are the ramifications of the vessels so much bigger as they originate in a bigger trunk, that is the principal ramifications; the same continues in the ramifications of the ramifications till the end*. Then, although unaware of the geometrical and mathematical significance of such observations, Leonardo's drawings illustrated and anticipated the modern

Figure 2. Different types of venous ramification according to Leonardo da Vinci (see text for a full description). From Vangensten et al. (1911-1916; volume IV, folio 8 recto). Library of Medicine and Surgery, and Pharmacy. University of Pisa.

concept of fractal formation and distribution of natural structures, such as rivers and blood vessels (Scianna et al., 2013). More recently, the "self-similarity logic" concept, dealing with a hierarchical multi-level organization in which very similar rules (logic) apply at each level, has been also proposed as a unitary scheme to describe many features of the formation of the vascular system and its remodeling processes (Guidolin et al., 2011).

In the Renaissance period, the father of modern anatomy, Andreas Vesalius (1514-1564), in his masterpiece *De Humani corporis fabrica* (On the Fabric of the Human Body) corrected the Galen's assumption that great blood vessels moved from liver to supply all parts of the body and doubted the existence of interventricular pores. In the same period, the philosopher and theologian Michael Servetus (1511-1553) and the anatomist Realdo Colombo (1516-1559) rediscovered the pulmonary circulation and firmly denied a communication between the two cardiac ventricles (Azizi et al; 2008; Limet, 2010; Aird, 2011).

Embryological studies favored the discovery of more dynamic features of the cardiovascular system. In the work *De foramine* (Foramen Ovale), Leonardo Botallo (1530-1587) provided a more complete description of the interatrial foramen ovale. His name was also associated with the ductus arteriosus, connecting the main pul-

monary artery to the proximal descending aorta. Giulio Cesare Aranzio (c. 1530-1589) dedicated some researches to the study of umbilical vessels and in the work *De humano foetu libellus* (Book on the Human Fetus) described the ductus venosus, which shunts the fetal umbilical vein blood flow directly to the inferior vena cava. Because of incorrect ideas about blood circulation, Girolamo Fabrici d'Acquapendente (c. 1533-1619) was not able to discover some features of the fetal vascular system. However, his embryological studies, published in *De formato foetu* (The Formed Fetus) (1600) and *De formatione ovi, et pulli tractatus accuratissimus* (Accurate Treatise on the Formation of the Egg and of the Chick) (1621), are noteworthy. He realized that after birth the umbilical cord and its vascular content dried up and disintegrated, having accomplished their function within the womb. In another work, *De venarum ostiolis* (Valves of Veins) (1603), he fully described anatomy and function of venous valves (Aird, 2011).

In the work *Peripateticarum quaestionum libri quinque* (Five Books of Peripatetic Questions), Andrea Cesalpino (1524-1603) introduced for the first time the concept of "circulation" applied to the cardiovascular system and also mentioned the presence of very small blood vessels, named *capillamenta* (hair-like vessels). But the important step in the history of the cardiovascular anatomy and physiology is represented by the notion of a systemic circulation of the blood, which is propelled by the heart, finally regarded as a muscular pump. The famous author of this mature account of the blood circulation as a closed circuit was William Harvey (1578-1657), who published his fundamental observations in the work *Exercitatio anatomica de motu cordis et sanguinis in animalibus* (An Anatomical Exercise on the Motion of the Heart and Blood in Living Beings) (Azizi et al; 2008; Aird, 2011).

Heart and blood vessels in the scientific revolution: mechanism and microscopic investigations

The French philosopher René Descartes (1596-1650) based his explanation of the world, including living beings, on a mechanistic model. Against Harvey's opinion, he postulated a heat theory to explain cardiac movements and blood circulation, through distillation, agitation, rarefaction and fermentation of particles (animal spirits), as reported in his work *Description du corps humain* (Description of the Human Body): *So I will say here that the heat in the heart is like the great spring or principle responsible for all the movements occurring in the machine*. Then, the development of a fetus was a mechanistic process, as well, according to an epigenetic model that included differentiation and growth (Heitsch, 2016). Concerning the development of the human body, Descartes described the parts of the body which are formed in the seminal material. The substance forming the blood also induced the development of the heart and subsequently the great artery originating from the heart to which it came back through another pathway: *As soon as the heart begins to form in this way, the rarefied blood leaving it makes its way in a straight line in the direction of least resistance, viz. towards the region of the body where the brain forms later on; and the path taken by the blood begins to form the upper part of the great artery. Now, because of the resistance produced by the parts of seminal material which it encounters, the blood does not travel very far in a straight line without being pushed back towards the heart along the same path by which it came. But it cannot return down this path, because the way is blocked by the new blood which*

the heart is producing. Then, in the same way blood vessels are formed: *I do not need to explain the formation of arteries and veins, because I have nothing else to say. Blood vessels are formed according to this general mechanism. When a little part of the seminal material reaches the heart, the pathway it creates is a vein, and the pathway created by the blood coming from the heart is an artery. When these blood vessels are far from each other, vein and artery appear separated, because the extremities of the artery are not visible anymore.*

Malpighi described the network of pulmonary capillaries that connect the small arteries to the small veins, then completing the closed circuit postulated by Harvey. However, this discovery did not fully clarify the vascular nature of capillary networks in particular organs, such as uterus, spleen and cavernous tissues in genitals. For example, some scientists, such as the above-mentioned Hunter and other anatomists, including Georges Cuvier (1769-1832), Friedrich Tiedemann (1781-1861), Bartolomeo Panizza (1785-1867), Ernst Heinrich Weber (1795-1878) and Pierre Augustin Béclard (1785-1825) recognized the vascular nature of the cavernous tissue. In the 18th century alternative hypotheses were also reported. For instance, in the case of erectile tissues of genitals, with particular attention to the male urethra, the presence of a non vascular spongy tissue with cellular texture (cellular theory) was postulated by some authors, such as Albrecht von Haller (1708-1777), Guichard Joseph Duverney (1648-1730), Herman Boerhaave (1668-1738) and Marie François Xavier Bichat (1771-1802). According to this theory, the cavernous tissue was conceived as consisting of a loose and elastic tissue arranged in several cellular cavities into which, during erection, blood was poured from the arteries, and from which it was afterwards removed by veins. Finally, thanks to improved injection techniques, Paolo Mascagni (1755-1815) and Alessandro Moreschi (1771-1826) provided accurate works on this subject, demonstrating the vascular nature of the cavernous bodies. Finally, in 1899 Victor Vecki von Gyurkovechky (1857-1938) confirmed the vascular theory, histologically demonstrated by the presence of endothelium: *These small hollow interspaces of the three corpora are coated with endothelium resembling that of the veins, and are consequently venous spaces* (Armocida and Natale, 2019).

As opposed to epigenesis (or neoformationism), preformationism developed in 17th century. According to this theory, as suggested by its name, living beings develop from a miniaturized preexisting form. Paradoxically, this approach became popular thanks to the first microscopic observations, when the presence of preformed organisms (*homunculi*) was believed in ova or spermatozoa. This theory was supported by important scientists of that time, such as Jan Swammerdam, Marcello Malpighi, Charles Bonnet, Albrecht von Haller and Lazzaro Spallanzani. Of course, such an approach simply admitted a pantographic growing of preformed organs and strongly limited the concept of formation of new tissues and organs from undifferentiated cells. Then, it is not surprising if the microscopist Antonie van Leeuwenhoek (1632-1723) observed *all manner of great and small vessels, so various and so numerous that I do not doubt that they be nerves, arteries and veins... And when I saw them, I felt convinced that, in no full grown body, are there any vessels which may not be found likewise in semen* (Friedman, 2008).

It should be remarked that a large part of the above-mentioned discoveries described blood vessels as static anatomical structures and said nothing about their formation and development, at least in the modern sense. Finally, in his fundamental *Theoria generationis* (Theory of Generations), a doctoral dissertation published in 1759, Cas-

par Friedrich Wolff (1734-1794), one of the founders of modern embryology, definitively affirmed the theory of epigenesis by demolishing the bases of preformationism, and showed that the organs in the embryo are formed successively from organized primitive tissues. He described the formation of veins (*De formatione venarum*) and arteries (*De formatione arteriarum*) and vessel branches (*Ramificatio venarum* and *Ramificatio arteriarum*). According to Wolff's model, the development of plants and animals was based on two main factors: the ability of organic fluids to solidify, and a mysterious organizing force named *vis essentialis* (essential force). At the moment of solidification, the movements of fluids into the new part lead to the formation of vesicles and vessels. More in depth, vesicles develop at an early step, when fluids move into the initially homogeneous secreted material, forming stationary pockets. Similarly, blood vessels are formed from the action of fluids moving through the homogeneous substance. Unlike plants, where vessels form and develop in parallel, in animals the fundamental substance is not rigid, allowing the development of branching blood vessels, which finally refer to the heart (Roe, 1979; Van Speybroeck et al., 2002). Wolff confirmed his ideas also in another work: *De formatione intestinorum* (On the Formation of the Bowel).

An important step in the history of the cardiovascular system is represented by the first *in vivo* description of blood vessel growth, which is attributed to the Scottish surgeon John Hunter (1728-1793), who showed that vascularization is an active process in tissues. In particular, in his book *A treatise on the blood, inflammation, and gunshot wounds*, published in 1794, he described the process of growth of new blood vessels, anticipating the modern concept of angiogenesis. Hunter observed an increased vascularization not only during the growth of young animals, but also in disease conditions and healing processes: *As a further proof that this is a general principle, we find that all growing parts are much more vascular than those that are come to their full growth; because growth is an operation beyond the simple support of the part: this is the reason why young animals are more vascular, than those that are full grown. This is not peculiar to the natural operation of growth, but applies also to disease, and restoration. Parts become vascular in inflammation; the callus, granulations, and new formed cutis, are much more vascular in the growing state, or when just formed, than afterwards; for we see them crowded with blood-vessels when growing, but when full grown, they begin to lose their visible vessels, and become not even so vascular as in the other neighbouring original parts, only retaining a sufficient number of vessels to carry on the simple oeconomy of the part; which would now seem to be less than in an original part. This is known by injections, when parts are in the growing state, or are just grown, and for some time after* (Stephenson et al., 2013; Lenzi et al., 2016; Natale et al., 2017; Bikfalvi, 2017; 2018).

Other important descriptions of vessel sprouting from pre-existing vessels, especially in inflammation and cancer tissues, were provided by different authors: in 1826 by Jacobus Ludovicus Conradus Schroeder van der Kolk (1797-1862) in *Observationes Anatomico-Pathologici et Practici Argumenti* (Anatomo-Pathological Observations and Practical Arguments), where new vessels in newly formed parts and in parasitical diseases were reported, and in what respect they differ from the natural structure; in 1844 by E.A. Platner in *Einige Beobachtungen über die Entwicklung der Kapillargefässe* (Some observations on the development of capillaries); in 1853 by J. Meyer in *Über die Neubildung von Blutgefässen* (On the Formation of New Blood Vessels); in 1856 by Christian Albert Theodor Billroth (1829-1894) in *Untersuchungen über die Entwicklung der Blutgefässe* (Studies on the Development of Blood Vessels) (Natale et al., 2017).

Angiogenesis in the 20th and 21st century

Angiogenesis is a term created to indicate the process by which the vascular tree grows by sprouting, cell division, migration, and assembly of endothelial cells derived from pre-existing vessels. Angiogenesis is not characterized by additional differentiation of endothelial cells, but rather by the reorganization of an existing vascular network in response to some angiogenic factors (Ribatti, 2014; Bautch and Caron, 2015; Ribatti et al. 2015). The research on angiogenesis has been usually tightly linked with the cancer field because of the importance of this process for the development of tumor masses. Thus, it is not a surprise that the main scientific achievements on neovascularization have been made looking at the cancer angiogenesis.

The beginning of angiogenesis era

As stated before, already in John Hunter's works the concept of angiogenesis was described, but not the term "angiogenesis" to indicate this process (Natale et al. 2017). Indeed, the debut of the term "angiogenesis" was due to Flint in 1900, who described the vascularization, and its development, of the adrenal gland referring to the work of previous scientists, such as Billroth, Thoma and Arnold (Flint, 1900) who traced the new formation of capillaries from those which already existed. Moreover, three years later Flint (1903) studied the vascularization and the blood vessel development of the submaxillary gland. Tumor vascularization started to be studied systematically by Goldman (1908). He used intra-arterial injections of bismuth diluted in oil to visualize the vasoproliferative reaction of an organ where a tumor is developing. Indeed, he described a marked capillary budding and new vessel formation particularly at the border of the neoplasm, also citing the work of other colleagues such as Hunter, Schroeder van der Kolk, and Broca. In the decade, Florence Sabin (1917) (Fig. 3) focused her research on the origin of blood vessels from endothelial cells that are already integrated into the primary vascular plexus of the embryo and subsequently proposed that in the early phase of embryonic angiogenesis endothelial cells are derived from precursor cells, the angioblasts (Sabin, 1920). A year later, Clark (1918) described the capillary sprouting in tadpole tails from capillaries with high flow.

However, the angiogenic process and the vascularization of tumors had the attention of various anatomists through the 20th century. As reported by Ribatti (2009) in his book on the history of tumor angiogenesis, during 1927, Lewis (1927) described the neovascularization of several rat tumors and observed that the vascular architecture of each tumor was different, leading to the conclusions that tumor environment greatly influence the morphological characteristics of the blood vessels, and that a common pattern could not be recognized. A great increase in the knowledge and description of angiogenesis was reached with the introduction by Sandison (1928) of the experimental use of a transparent chamber that could be inserted into the ear of rabbits. This experimental device allowed the microscopic observation of living tissues underneath a glass coverslip. Successively, Clark and colleagues, thank to this experimental tool, described the morphological characteristics of blood (Clark et al., 1931) and lymphatic vessels (Clark and Clark, 1932), using also contrast media, and the changes of the newly grown vessels over a period of months (Clark et al., 1931).

Figure 3. A photograph of Florence Sabin (from the Profiles in Science of the US National Library of Medicine).

Moreover, he left detailed observations on the growth of blood capillaries in living animals, showing that capillary sprouts had adventitial cells (Clark and Clark, 1939), opening the field to the subsequent studies of Ide et al. (1939) who used the transparent chambers to investigate the relationship between the growth of rabbit epithelioma and its vascular supply. These scientists observed that the tumor mass increased with a rapid and extensive formation of new vessels that were necessary to its development. Moreover, they firstly suggested that tumors might release factors capable of stimulating the blood neovascularization growth. In 1941, Green (1941) demonstrated that the vascularization was necessary to the growth of a transplanted tumor, whereas few years later, Algire and Chalkley (1945) firstly described that the progression of malignancies could continuously elicit new capillaries from the host. Moreover, they also quantified the number of blood vessels with daily counts, comparing them to the changes in tumor size and concluding that the growth of a tumor is closely connected with the development of an intrinsic vascular network. This feature was emphasized by Rondoni (1946), an Italian pathologist, who described that a tumor acts both angioplastically and angiotactically, promoting both the formation of new vessels and the attraction of vascular capillaries.

The evolution of angiogenesis concept

In 1948, Michaelson (1948a) described the vascular morphogenesis of the retina and hypothesized that a diffusible "factor X" produced by the retina was responsible for retina neovascularization in proliferative diabetic retinopathy [Michaelson, 1948b]. Interestingly, in 1956, Merwin and Algire (1956) found out that the vasoproliferative response to normal or neoplastic tissues transplanted into muscles was not different in terms of the time of onset of new blood vessels but was significantly divergent in terms of intensity and influenced by the distance between the implant and host's vessels: tumors had a longer activity range. Another fundamental step to the comprehension of the presence of a diffusible factor stimulating the new capillaries was brought by Greenblatt and Shubik (1968) using a Millipore chamber into a hamster cheek pouch. Indeed, because the pores of the chamber were permeable to the tumor interstitial fluid but not to tumor cells, the new blood vessels from the host were formed in any case, without any contact with cancer cells. Based on this evidence, the authors hypothesized the presence of a diffusible factor responsible for the neovascularization. Moreover, Ehrmann and Knoth (1968) in the same year confirmed these data with tumor fragments laid on Millipore filters planted on a Chick Chorioallantoic membrane (CAM). In the 1970s, Gimbrone and Gullino (1976), using the rodent mammary gland, demonstrated that a resting gland had not any angiogenic capacity, whereas the presence of a neoplastic transformation induced angiogenesis.

The perspective in the field of angiogenesis changed radically with the pivotal article published in the New England Journal of Medicine (Folkman, 1971) by the surgeon Judah Folkman who reported several ideas that drastically modified the perception of angiogenic process in the scientific community, such as the essential role played by angiogenesis in cancer growth, the secretion by tumour cells of diffusible angiogenic molecules, the possibility of tumour dormancy due to the blocked angiogenesis, the development of the concept of "antiangiogenesis", the prediction of the future discovery of angiogenesis inhibitors, and finally the concept that an antibody targeting a tumour angiogenic factor (TAF) could be an anticancer drug (Fig. 4). This last idea inspired a completely new field in anticancer therapy that is still pursued by numerous scientists and oncologists (Jayson et al., 2016) with drugs such as bevacizumab, an anti-vascular endothelial growth factor (VEGF) antibody, conceived in the fundamental work by Napoleone Ferrara (Ferrara et al., 2004). In the subsequent years, Folkman provided numerous evidences supporting his initial hypothesis, also developing fundamental assays to study the angiogenic process. In 1972, his team demonstrated that *in vivo* tumour dormancy was obtained by prevention of neovascularisation (Gimbrone et al. 1972) and, a year later, he was the first, in collaboration with his group, to successfully grow and passage vascular endothelial cells *in vitro* (Gimbrone et al., 1973), an experimental technique that permitted the future development of all the antiangiogenic drugs in the next four decades. Moreover, angiogenesis was firstly observed *in vitro* by Folkman and Haudenschild (1980). After long-term culture of capillary endothelial cells, they observed the spontaneous organization of these cells into capillary-like structures with the presence of a lumen. *In vitro* three-dimensional models of endothelial cells grown in collagen gels have provided great steps in the knowledge of angiogenesis. As an example, Montesano et al. (1986) observed that fibroblast growth factor induced endothelial cells to form

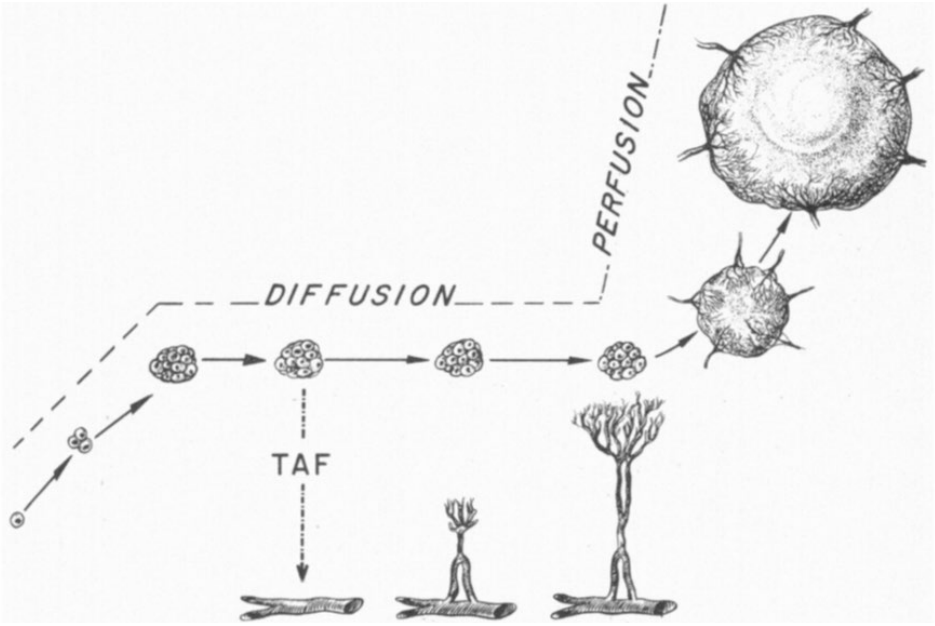

Figure 4. Original figure n. 2 of the pivotal article published by Folkman (1971). The figure describes the concept that most solid tumors require vascularization for their growth and that tumor angiogenesis factor (TAF) may be the mediator of neovascularization.

capillary-like structures in collagen gels, showing the *in vitro* angiogenic activity of this cytokine.

The exciting era of pro- and anti-angiogenic factor discoveries

Despite the work by Greenblatt and Shubik (1968), until the '70s it was largely accepted that tumors did not secrete specific angiogenic proteins. The conventional thinking was that tumor vasculature derived from the inflammatory reaction of necrotic cancer cells (Ribatti, 2009). Folkman (1971), as described above, hypothesized the presence of a soluble factor and in the same year he isolated a tumor fraction responsible for the angiogenesis process (Folkman et al., 1971). Indeed, the homogenate of a murine breast cancer was fractioned by gel-filtration and found out that a fraction of about 10,000 KDa had the strongest angiogenic activity. This active fraction was subsequently named "tumor angiogenesis factor" (TAF). This fraction induced vasoproliferative responses in different *in vivo* models such as CAM or rabbit cornea, and *in vitro* on cultured endothelial cells (Weiss et al., 1979).

Gospodarowicz (1975) found that the pituitary gland contains a potent agent for cell growth, although he did not identify the factor. However, he demonstrated that this putative factor induced fibroblast cell growth and hence he called it fibroblast growth factor (FGF) (Gospodarowicz and Moran, 1975). In 1978, he also found out that the activity of the FGF was not restricted only to fibroblasts but also to other

cell types, including endothelial cells (Gospodarowicz et al., 1978). Later, Shing et al. (1984) reported the isolation and the purification of FGF, initially identified by Gospodarowicz, the first factor that specifically stimulated the growth of endothelial cells. Moreover, the same group demonstrated that this factor was able to stimulate new vessel growth *in vivo* in the CAM assay (Shing et al., 1985).

Another important step forward to the knowledge of pro-angiogenic factors was the work of Dvorak and colleagues at the beginning of the '80s. Indeed, he demonstrated that vascular hyperpermeability to fibrinogen and other plasma protein was a common feature of many animal and human tumors (Dvorak et al., 1981; 1983; 1984). Dvorak et al. (1979) found out that supernatants from tumor cells generated blue spot to extravasated Evans blue, whereas normal cells did not. Dvorak named this tumor supernatant permeabilizing activity as vascular permeability factor (VPF) (Senger et al., 1983). Moreover, the team of Dvorak showed that VPF was a macromolecule and inactivated by the heat and proteases whereas the inhibition of protein synthesis declined its secretion (Senger et al., 1983). Senger et al. (1983) purified VPF, demonstrating that it was a 34-43kDa dimeric protein. Subsequently, Connolly et al. (1989) of the Monsanto Company showed that VPF was also an endothelial mitogen *in vitro* and an angiogenic factor *in vivo*.

In the same year, Ferrara and Henzel (1989) reported the isolation, of a diffusible endothelial cell-specific mitogen from a medium conditioned by bovine pituitary follicular cells, which they called "vascular endothelial growth factor" (VEGF). Later, they reported the isolation of cDNA clones of different isoforms of the protein with clear angiogenic characteristics (Leung et al., 1989). In 1992, the Ferrara's laboratory in collaboration with the University of California at San Francisco discovered the VEGF receptor-1 (de Vries et al., 1992) whose expression was regulated by hypoxia (Gerber et al., 1997). Moreover, in 1996 both Ferrara et al. (1996) and Carmeliet et al. (1996) demonstrated the essential role of VEGF in embryonic vasculogenesis and angiogenesis in the mouse. Indeed, the inactivation of just a single allele of VEGF resulted in the embryonic lethality with a number of developmental anomalies of the cardiovascular system.

Over the years, five VEGF-related genes have been identified, as well as five different related receptor tyrosine kinases (Uccelli et al., 2019), becoming the main known pro-angiogenic factor. The VEGF was then recognized to be the same factor discovered, but not sequenced, by Dvorak and colleagues.

In 1996, a novel family of angiogenic factors, called as Angiopoietins (Ang), has been identified by the group of Yancopoulous (Davis et al., 1996; Maisonpierre et al., 1997). Ang1 is a potent angiogenic growth factor signalling through its receptor Tie2, whereas Ang2 was initially identified as a vascular disruptive agent with antagonistic activity through the same receptor. Recent data, instead, demonstrate that Ang2 has context-dependent agonist activities (Akwii et al., 2019).

Later, the scientists discovered that the factors able to activate and regulate angiogenesis were far more than just the few hypothesized by Folkman and other scientists, including for example platelet-derived growth factors, placenta growth factor, insulin-like growth factors, hepatocyte growth factor, hypoxia-inducible factor-1 α and β, transforming growth factor α and β, tumor necrosis factor α, interleukins -1 β, -3, -6, -8, neuropilin 1 and 2, angiogenin, adrenomedullin, and stromal cell-derived factor-1 (Natale and Bocci, 2018).

Besides the pro-angiogenic factors, during the years the scientists discovered also numerous endogenous inhibitors of angiogenesis and their list continues to grow with new discovered molecules (Rao et al.. 2015). Many proteins have been identified as endogenous angiogenesis inhibitors including thrombospondins (TSP)-1 and −2, pigment epithelial derived factor, platelet factor-4, and various interleukins and interferons. Collagen and plasminogen fragments have been also identified as angiogenesis inhibitors such as angiostatin (fragment of plasminogen), endostatin (fragment of collagen XVIII) and tumstatin (fragment of collagen IV) (Natale and Bocci, 2018). Among these inhibitors, at least eleven endogenous angiogenesis inhibitors were identified or discovered in the Folkman's laboratory in almost 25 years (Folkman, 2008). In 1982 Folkman and Taylor (1982) recognized that protamine and platelet factor 4 inhibited angiogenesis and in 1985 he reported a new class of corticosteroids, called "angiostatic" steroids (Crum et al., 1985). Moreover, O'Reilly and colleagues discovered in 1994 the antiangiogenic and antitumor activity of angiostatin (O'Reilly et al., 1994) and later of endostatin (O'Reilly et al., 1997).

TSP-1 was the first protein to be identified as an endogenous inhibitor of angiogenesis by Good et al. (1990). In the same year, Taraboletti et al. (1990) demonstrated that this heparin-binding protein stored in the extracellular matrix was able to inhibit endothelial cell proliferation of different tissues. Moreover, Jack Lawler (2002) obtained TSP-1 null mice and showed that tumors grew significantly faster in these mice. Bocci et al. (2003) displayed the role of TSP-1 in the antitumor effect of metronomic chemotherapy, a therapeutic approach able to inhibit angiogenesis.

The last developments in angiogenesis field of research

However, not only solid cancers depend on angiogenesis for their growth. In 1994, for the first time, Vacca et al. (1994) demonstrated the angiogenesis involvement in haematological malignancies. These authors described the presence of bone marrow angiogenesis in multiple myeloma with a high correlation between the extent of neovascularization and plasma cell proliferation. A year later, Klein et al. (1995) confirmed these data. Moreover, Ribatti et al. (1996) firstly showed the bone marrow angiogenesis also in B cell non-Hodgkin's lymphomas, whereas Perez-Atayde et al. (1997) brought the first evidence of increased bone marrow microvessel density in acute lymphocytic leukemia.

In 1996, Zimrin et al. (1996) suggested for the first time that Jagged-Notch signaling was able to regulate FGF-induced endothelial cell migration *in vitro*, an early and key event during the process of angiogenesis. Five years later, Mailhos et al. (2001) reported the expression of Delta4 in arterial endothelium during mouse embryogenesis and in the endothelium of tumor blood vessels. The authors of this study concluded that Delta4 and the Notch signalling pathway could have a primary role in angiogenesis and suggested them as possible new targets for antiangiogenic tumor therapy. Shawber and Kitajewski (2004) stated that Notch genes were highly expressed in the vasculature suggesting an important role for Notch in guiding endothelial cells to form the vasculature. Indeed, studies in zebrafish, mice and humans indicated that Notch works in conjunction with other angiogenic pathways to pattern and stabilize the vasculature. Recently, Pan et al. (2019) revealed the inhibitory role of TSP-2 on cell invasion, migration and angiogenesis in the development of medulloblastoma *via*

blockade of the Notch signaling pathway, suggesting its potential as a new treatment target for this brain tumor.

In 1997, Asahara et al. (1997) reported the isolation of putative endothelial progenitor cells from human peripheral blood, on the basis of cell-surface expression of CD34 and other endothelial markers. These authors showed that hematopoietic cells differentiate into endothelial cells *in vitro* and *in vivo*. The importance of endothelial progenitor cells in the process of angiogenesis, and in particular of tumor angiogenesis, has been discussed during the years but, in 2008, Gao et al. (2008) demonstrated that also a low percentage of incorporated endothelial progenitors in the vasculature of tumors is sufficient and necessary for the conversion of avascular micrometastases to progressive metastatic tumors.

In 1999, Betsholtz's group described the mechanisms of pericyte recruitment to newly formed blood vessels with the pre-eminent role of PDGF-B and PDGFR-beta (Hellstrom et al., 1999), and subsequently discovered that specific cells atop the nascent vessel guide the growth of the vascular tubes (TIP cells) with the VEGF stimulus (Gerhardt et al., 2003). Few years later, different authors described that the vessel lumen formation is an active process which requires at least one endothelial cell (Kamei et al., 2006; Blum et al., 2008; Strilic et al., 2009). More recently, Carmeliet's team discovered that the vessel sprouting requires a specific metabolism and in particular the PFKFB3-driven glycolysis (De Bock et al., 2013).

In 1999 Maniotis et al. (1999) coined the term vasculogenic mimicry (VM) when they reported that human melanoma cells were able to form vascular channels observing sections from aggressive human intraocular and metastatic melanomas. The word vasculogenic was referred to the formation of blood supply system by tumor cells rather than endothelial cells, which is independent on typical modes of angiogenesis. Although the data by Maniotis and collaborators were initially harshly disputed by Mc Donald et al. (2000), later on VM has been shown and deeply described in various cancers including melanoma, breast and lung cancer, ovarian cancer, osteosarcoma, gastric cancer, bladder cancer, hepatocellular cancer, and colorectal cancer (Azad et al., 2019). Indeed, high tumor VM is associated with a high tumor grade, shorter survival, invasion, metastasis and poor prognosis (Delgado-Bellido et al., 2017).

Another important concept that has acquired recent interest in the field of neovascularization is the vessel co-option (or vascular co-option): "*a mechanism by which tumors obtain a blood supply by hijacking the existing vasculature and tumor cells migrate along the vessels of the host organ*" (Donnem et al., 2013). Pezzella et al. (1997) were the first to report a non-small cell lung cancer growing with no morphological evidence of neoangiogenesis but only exploiting normal tissue vessels already present. Indeed, many studies have confirmed that the microcirculation of some human tumors may be provided by nonsprouting vessels and that a variety of tumors can grow and metastasize without angiogenesis (Krishna Priya et al., 2016). Vessel co-option may be found in many tumors but especially in highly vascularized tissues such as brain, lung, and liver (Donnem et al., 2013). Recently, vessel co-option has been involved into the potential explanation of antiangiogenic drug resistance and it has been suggested the tumor progression can only be stopped by combination therapies that block both angiogenesis and cooption (Voutouri et al., 2019).

Recently, a new important direction of the research in this field is the study of the relation between the vascular system with immunology. Indeed, the work by two

laboratories focused on the importance of specialized vessels called high endothelial venules (HEV) in tumors to trigger anti-cancer immunity (Martinet et al., 2011; Allen et al., 2017). The tumor vasculature-immune interdependency, in the perspective of an anti-tumor response, could be an important step into a new approach to antiangiogenic therapy with the immunotherapy combination using anti-PDL1 because it is able to stimulate the number of HEV vessels in tumors (Allen et al., 2017).

In conclusion, the research on angiogenesis in the last fifty years has been tightly linked with cancer research, and more recently with anti-angiogenic drug development. Thus, in a recent review Bikfalvi (2017) placed the important question about the future of the field and which will be the landscape of angiogenesis research in the next 20 years. Indeed, industry seems not investing any additional efforts in the development of anti-angiogenic compounds in cancer and this may delay future discoveries in this fundamental field for different areas of biology and medicine.

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