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Halpern SD, Ubel PA, Caplan AL. Solid-organ transplantation in HIV-infected patients. N Engl J Med. 2002;347:284-7.

Books and Monographs

Author(s) and editor(s)

Breedlove GK, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services; 2001.

Chapter in a book

Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. p. 93-113.

Electronic materials

Homepage/Web site

Cancer-Pain.org [Internet]. New York: Association of Cancer Online Resources, Inc.; c2000-01 [updated 2002 May 16; cited 2002 Jul 9]. Available from: http://www.cancer-pain.org/.

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EDITORIAL

Experiments in medical research

F Passariello[1]

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Experimental research is generally considered predominant in Medicine, in comparison with other articles regarding theories, reviews, etc..

Medical journals as well as many meeting organizations, nowadays prefer scientific contributes, which fit the pre-requisite of the well-known IMRaD[1] sequence: introduction, aims, materials and methods, results, discussion and conclusion. This strict scheme is ubiquitary, even if often erroneously applied to all types of medical research[2].

The current issue of JTAVR guests 2 original articles, which are compliant with this accepted standard scheme, presenting relevant data about compression therapy (Rastel) and venous diagnostics (Ermini).

The paper of Rastel deals with the proposal of a new device, able to measure locally the "ex-vivo" pressure of compression stockings, when donned on the leg. The aim is to replace the time consuming but highly reliable, normalized measurement in a textile laboratory with a less precise hybrid "ex-vivo" method, which is however even more precise than the "in-vivo" measurements, gathered with wearable devices.

The paper of Ermini practically shows how a venous dynamic test, which is daily practised in most vascular labs since more than 20 years, can gain a reliable corroboration, when submitted to validation through a strict experimental protocol against a generally accepted manoeuvre like the squeezing test. In this way, daily practice enters the promising world of scientific validation.

Although there are a lot of useful papers, which explain in clear words the way to successfully write a scientific article, let me remind you all some non-written rules, which can be of paramount importance in setting the value of your manuscript.

Both theoretical and experimental research have their own well-defined specificity. It is therefore essential to set clearly the category of the manuscript, avoiding mixing in the same text a new theory and a new experiment. Practically both deserve a separate article, with a deep description of their details.

An experimental article should describe the theoretical and literature premises in the introduction, justifying the aims of the paper. Practically, *from Theory to Experiment*.

In the same time, it should show any changes in the theoretical frames in the discussion, *what the paper adds* to the current literature. Practically, *from Experiment to Theory*.

Methods and Results should be self-contained, i.e. they should not have direct relationship (apart of references) with the other parts of the paper.

There must be a continuous osmosis between the experimental research and the models and intepretations of literature, even if already accepted or still in evaluation.

An experimental research, which does not refer to literature or to a theoretical frame, is just a *blind research*, which drives you nowhere.

Finally, an experimental research should suggest *how to go on* in the neverending process of scientific research.

Following these suggestions, we will find us all together *after the same rainbow's end as research friends*.

Fausto Passariello

Editor in Chief

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HUMAN-ANIMAL INTERACTION BOOK REVIEW

Humans and Animals: Issues in Ethics

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Abstract In the last decades of the twentieth century the question of relationship between humans and animals has become a very important area of Applied Ethics. Indeed, starting from seventies the specism (considered as form of discriminatory of non-human animals) has been put to the center of moral reflection mainly thanks to Peter Singer and Tom Regan. The debate is still in progress and have been added other philosophical positions, as an example the Ethics of care (Rosalynd Hursthouse) and *Egalitarianism* (Ingmar Persson). Therefore, Pollo's text represents a valuable contribution of sentimentalism ethics to modern Animal ethics. The author's analysis does not want to be exhaustive, but it want to be instrument to reflect on ethical questions between humans and animals.

Keywords Animal ethics, Moral sentimentalist, Animal welfare, animal Testing, Moral progress

The privileged object of this work[1], as defined in a programmatic way in the introduction, focuses on the relations human beings have with animals (not humans) in daily life. Pollo's text, that is part of the sentimentalist tradition in ethics, aims at being a contribution to the development of Animal Ethics in common life. The book is dedicated to Eugenio Lecaldano, who is among the major exponents of this tradition in the Italian philosophical panorama. In complete agreement with this tradition, the author wants to investigate how feelings, simpathy and imagination can develop and the maintain moral reflection on the interaction between humans and animals. The book does this through a dense and constant comparison with the important theoretical and regulatory frameworks defined by Peter Singer and Tom Regan, the pioneers of modern antispecism.

Umani e animali, therefore, finds its place across the Italian and international specialized debate and it does so suggesting an alternative and, at the same time, fascinating point of view. Our moral reflection towards animals needs to proceed in constant agreement with everyday experience, since this latter should be given priority compared to the theoretical philosophical reflection. This methodological assumption represents the central theme of the essay and it embodies its main turning point. This type of framework, however, as advised by the author himself, does not want to suggest that the theoretical rationalistic analyses must be abandoned, but wants to affirm that the two need to be integrated by a more immediate dimension, as close as possible to what we are as human beings, belonging to the Homo Sapiens species.

In this context, we find the most performative aspect of the book: the variety of relationships we establish with animals and the daily direct and indirect interactions that we maintain with them are an integral part of our cultural and biological identity and therefore they should represent the starting point for the moral reflection on Animal Ethics.

The text can be ideally divided into three parts that correspond to the three objectives the author resolves to achieve: to offer an informative picture of some "facts" concerning the relationships between Homo sapiens and the other animal species (Ch 1); to illustrate how the research field of contemporary Animal Ethics originated through the analysis of some theoretical positions of the past (Ch 2, 3 and 4); finally, to discuss, from an alternative point of view (compared to the dominant concept), three big categories of interactions between humans and animals (nutrition Ch 5; use of animals for biomedical research Ch. 6; safeguard of wild animals Ch. 7).

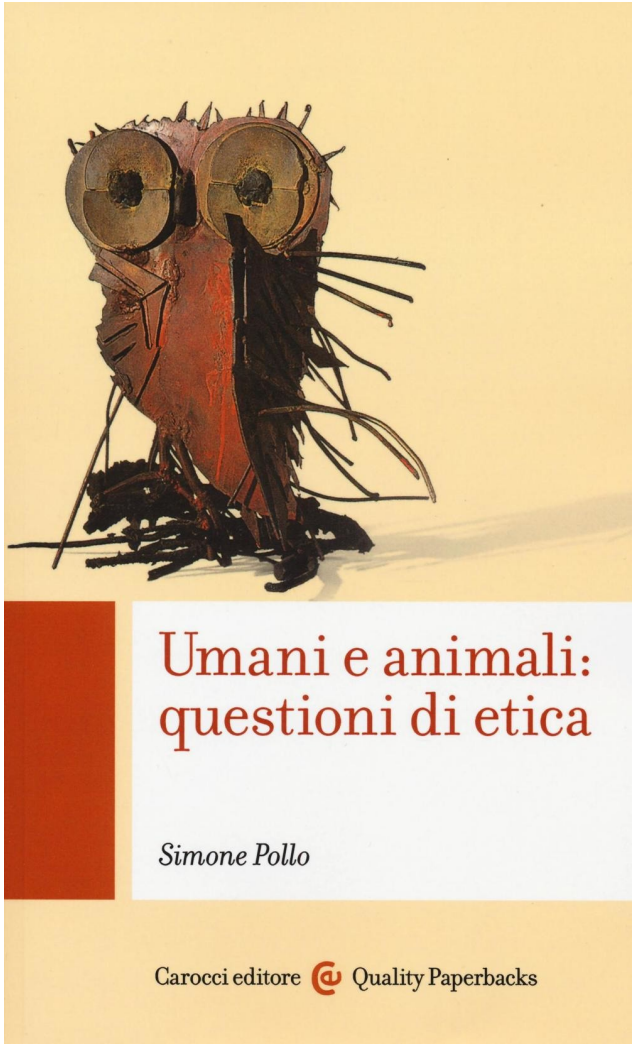

Figure 1 - Simone Pollo. Umani e animali: questioni di etica.

As for the first point, the idea (or the fact) that the author wants to support is contained in the following formula: "we are humans thanks to the animals" (p.17).

Through an excursus of the evolutionary and historical path of Homo Sapiens, the author provides solid proof supporting the idea that our form-of-life, as it is today, is inextricably connected to the life of non-human animals. This "fact" should be analysed, in Pollo's perspective, starting from the central mechanisms of "ecosystem" and "coevolution". The interdependence of the living species and their interactions, that lead to a continuous modification of the specific traits in a continuous process of reciprocal adaptation, are at the base of the genetic characteristics that we can observe in human beings nowadays. Human beings, hence, are this way because during their biological evolution they have been interacting with other non-human animals and they have been breeding them, eating them, etc. However, the author sees a possible fundamental turning point for evolution mainly in the old practice of domestication: it is through this experience that human beings would have started to develop the ability to "understand" animals; this would have initiated a path that, through genetic transmission, lead to the development of the ability to understand others' minds, with all the ethical and historical implications entailed.

Another fundamental point of Pollo's analysis is the acquisition of Darwin's theory of evolution, interpreted as the theoretical-empirical corpus denominated "New synthesis"[2]. According to the author, this theory represents the "turning point" that works as and create a divide between a "before" and an "after" in the field of Animal Ethics. Indeed, starting from Darwin, the ontologically-based approach proposed by René Descartes on the discontinuity between animals and humans will become unsustainable. This assumption is one of the two "theoretical requirements" the author defines along the book.

The second is the need drawing attention to the variety of positions presented in the first place by Darwin himself and represents the second objective the author is determined to achieve. Comparing thinkers like Aristotle, Saint Thomas Aquinas, Kant and Porphyry of Tyre, the author demonstrates how reflection on the relationships with animals is well established in the tradition of Western thought and how Animal Ethics, as it is structured today, represents the last chapter of a richly diversified path that has its roots in the past (in regards to this, Pollo also reminds us that Animal Ethics has developed in conjunction with the arrival of the feminist and anti-racist movements of the '60s and '70s).

However, the most radical thought/critique is found in the two following chapters (third and fourth) and it deals with the core of the anti-speciesist ideas proposed by Peter Singer, Tom Regan and Gary Francione. Pollo offers and alternative perspective to the theoretical normative ones of the two fathers of *Animal Liberation*. Where *Animal Liberation* affirms that "rationality" must take on a central role in the development on Animal Ethics, Pollo presents a sentimentalist perspective that places emotions and sympathy at the centre of moral life. It is a "sentimentalist anti-anthropocentrism", conscious of the fact that change is possible only from a human point of view, with its potentialities and limits. Indeed, the rationalistic ethics of Singer and Regan on its own might not be enough to overcome those obstacles inherent to human beings that were well highlighted by moral psychology: the partial inability of human beings to follow the rules laid out in theory and the fact that the cultural context in which we live and our biological structure itself are profoundly linked (because inherited) to the life of other animal species. For this reason, Pollo's perspective moves from the "bottom" of experience and from its "inside" and aims to underline how

the forms of interactions with other species can become the centre of moral reflection.

Starting from this focal point, Pollo criticizes analyse Gary Francione's concept, according to whom all relationships with animals (including domestic ones) must be considered as forms of exploitation and slavery and should therefore be suppressed. The risks coming from such a view, according to Pollo, are the following: under defining the relevant aspects of moral experience and, as in the monist theories of Singer and Regan, leaving little space to the variety of possible situations that can lead human beings to empathize with the other sentient beings.

At this point in the text, after expressing his method and the direction he wants to give to his reflection on Animal Ethics (following the one proposed/developed by the neo-Humean philosopher Mary Midgley), the author takes on the analysis of three big categories of interaction between humans and animals (this is the third purpose of the book). Consistent with his theoretical approach, the discussion will not end with argumentations in favour of *animal liberation*: indeed, it is important to remember that Pollo's contribution wants to be an opportunity to reflect on the ways "human beings shape their behaviour towards animals in the different contexts in which they come into contact with them in their daily life" (p.84).

The first big category the author focuses on is the use of animals for food: the essential precondition for every discussion on morality in this background concerns our biological and cultural inheritance, in which the presence of food of animal origin is "inevitable" (p.89). After analysing Singer and Regan's positions on the subject, respectively based on the Utilitarian paradigm and on the Rights theory, Pollo's analysis emphasizes the idea that the public acknowledgment of some form of rights (in this case for animals) represents the end point of moral reflections and transformations that act, first of all, on the moral agent at an individual level. Therefore, referring to John Mill's concept of "self-perfectioning", Pollo's idea of challenging nutritional behaviours presents itself "as a criticism on one's own identity and in the possibility of recognising oneself in those transmitted habits" (p.97). The characteristics that need to be nurtured are the following: attention to one's own personality, questioning of oneself and inclination towards moral growth. These, indeed, can expand moral recognition of animals, leading to an informed and lasting choice towards a vegan or vegetarian diet.

A very interesting aspect of this analysis is the fact that it intersects with feminist reflection and the Gender studies. Referring to Carol Adam's analysis of philosophy (2013), the author states that the culinary culture in our society includes some elements of male sexism and promotes forms of gender discrimination (p.100). This position is confirmed by the important study by Erika Cudworth who emphasizes two main turning points relating to carnivorous diets. The first is the presence, in western society, of a type of hierarchy of food in which red meat consumption is usually associated with manliness while white meat and dairy products are usually related to femininity. The second point concerns the way in which we prepare meat: roasting or grilling red meat, for example, is the favourite cooking method for men because the meat remains rare, recalling those myths that narrate how manliness and strength come from the animal blood. Boiling or stewing meat, on the contrary, seems to remind a more domestic and feminine context[3]. In any case, Pollo's intention is to underline the transformative potential that a vegetarian choice could prompt on ourselves and, therefore, on our environment and society.

Our interactions with "wild animals", that is those animals "whose existence does not depend on steady processes of domestication and selection" (p.118), represent another aspect of our life that is discussed by the author. While talking about nutrition, the discussion proceeded on the level of individual behaviour where the idea of *animal welfare* turned out to be the most functional. When the attention shifts to the interaction with wild animals, the level of analysis is that of social behaviour and the reflection revolves around the idea of "respect", considered as "moral acknowledgment and approval of the individuality and freedom of other subjects" (p.122). Indeed the author criticizes the dominant views of Martha Nussbaum on justice and of Singer on utilitarianism for not having fully absorbed the lesson coming from the Darwinian theory. This would be the essential missing part in the two theoretical positions analysed by the author that made them move, according to Pollo, towards the field of the metaphysical foundations of Judeo-Christian anthropocentrism, even if not sharing with it its ontological presuppositions. The acquisition of the theory of evolution, instead, would place human beings in a non-central position compared to the other sentient beings (and to the "natural" world) and, as a consequence, would lead society to make some critical considerations towards the practices of "preservation" of the ecosystems and of "wild" animals that originated precisely from the concept of "respect". On the theoretical level, a comparison between Pollo's approach and that of some bioethicists in favour of moral enhancement of non-human animals would be intellectually stimulating[4].

The use of animals for biomedical research is the third area of interaction discussed in the text and, undoubtedly, represents the most sensitive one although, as demonstrated through data by the author himself, it is the one with the smallest number of animals involved and especially considerably lower than, for example, the number of those used for food.

The use of animals for experimentation is an ancient practice dating back to the VI century a.C., that is when science as such was born in classical Greece. This practice is well established in the history of scientific research: this is the starting point, according to the author, for the analysis of its moral acceptability. Medicine has indeed reached extraordinary results in the last centuries, mainly thanks to experimentations on animals. Our form of life, therefore, is deeply linked to medical science and its results. Moreover, if referring to food and to the "preservation" of wildlife, the theoretical and practical path is definitely smoother (in principle, we can all do without steaks, creams, etc) and it becomes more difficult when what is at stake are the interests of life and death of human beings.

Reaching the core of the philosophical reflection in this specific context, Pollo discusses Singer's position. In accordance with the utilitarian approach, Singer's perspective shows how, in specific circumstances, the advantage of a certain number of sentient beings belonging to any species can justify the use of other sentient beings for experimental purposes. Pollo also discusses Regan's theory of rights that, on the contrary, categorically denies the morality of the use of sentient beings for this purpose. In both instances, the aim is to argue "in favour of a radical reform of science, suppressing de facto any invasive use of animals" (p.170). At this point, the author discusses if and how these approaches in the field of biomedical experimentation can actually prove to be effective. Whilst the approach of "perfect equality", peculiar to Regan's theory of rights, cannot bring convincing results, Singer's utilitarian approach, on the contrary, turns out to be the one that most complies with the changes in animal testing and therefore with the search of alternative methods not requiring the use of animals, a reduction in number of animals involved and a bigger attention to their welfare (as expected in the "model of the three Rs"). However, this approach presents some limits that are well described by the author, among which some stand out: the "conservation of the preferences of the species" and the uncertainty of positive outcomes in the ongoing research (pp. 110-111).

To limit an excessive "simplification" of these positions, Pollo's, in accordance with his chosen approach, places the analysis in the reality of everyday research. Indeed, the curve outlined by the author is in agreement with the genealogy of a Humean - conventionalist type of society, according to which sociality is built around forms of cooperation and reciprocity among humans and justice is the result of the stratification of regulations that are not always intentional (p. 109). This means that social critical responses to animal testing ("vivisection" is an unsuitable term for the contemporary context) will necessarily have to interact with the "specific contexts", that is with the scientific community. Concepts like "trust", "authority" and "truth" are an essential part of scientific research and, therefore, Pollo states that ethics does not have to "moralize" science, in opposition to what Singer and Regan's monist theories would support. The inclusion of animals in a broader sphere of moral reflection and the reconsideration of their use in research cannot be dictated by external regulations (such as European legislations, etc.), but these latter should be considered as sources for the promotion of moral reflection itself and of animal welfare, and they should be made available to researchers and technicians, that is to those who work in this context on a daily basis.

The effectiveness of this type of approach is confirmed, for example, in the case of the 27 macaques destined to research and recently released by the Health Research Consortium of Padova. The active role of the Consortium itself, together with the University of Padova and the Zooprophyilactic Institute of Venice, in the release of the animals has also been publicly praised by LAV[5], a particularly active animal rights association that fully opposes the use of animals for research.

In the conclusion of the book the author tackles the issue of "moral progress" in our society. The notion of "moral progress" is undoubtedly problematic and, as explained by Pollo, there are two ways to interpret it. The first takes inspiration from Dale Jamieson's formulation and here we can find the rationalistic concepts analysed in the previous pages of the text. According to this interpretation, "moral progress" would represent "the realization, in this world, of the process of accumulation of a particular value or good" (p.131). The second possible interpretation is the one outlined by the author himself in a previous article[6] and described as "the increase of moral reflexive skills in humans" (p.131). In this latter interpretation there is less stress on the individual adaptation to a normative model and more attention is being placed, instead, to the richness of moral experience in daily life. The two concepts, however, are not alternatives, the author says, but they need to intertwine and complement each other in order to contain the constant risk of falling into "moralism".

As a whole, in conclusion, Pollo's contribution to Animal Ethics represents a useful instrument of reflection for philosophers, animal rights activists, researchers and for all those interested in the ethical debate of the relations between humans and animals.

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The discovery of lymphatic system as a turning point in medical knowledge: Aselli, Pecquet and the end of hepatocentrism

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Abstract In this paper, I would like to analyse the impact of the discovery of lymphatic system on the development of the modern conception of human body. The discovery of lymphatics, as that of blood circulation, has in fact questioned important tenets of Galen's anatomo-physiology. Galen defended a 'dualistic conception' of the blood: he distinguished two different systems, the hepatic-venous system and the cardio-arterial one. The liver played a pivotal role because it was believed to transform the chyle received by the portal vein into venous blood. The discovery of lymphatics challenged this view: 17th-century anatomical dissections and experiments, starting with the discovery of milky veins by Gaspare Aselli (1581-1625) and the studies on thoracic duct by Jean Pecquet (1622-1674), irrefutably showed that the chyle does not pour out in the liver and that, consequently, the liver does not produce blood.

Keywords lymphatic system, chyliferous vessels, hepatocentrism, Gaspare Aselli, Jean Pecquet

Introduction

The history of the lymphatic system has recently caught the attention of both historians of medicine and physicians (phlebologists, lymphologists, haematologists, and so on), particularly those interested in the historical, methodological and ethical issues related to their discipline. Over the years, numerous scholarly medical journals — among others, *Lymphology*, *Clinical Anatomy*, *British Journal of Haematology*, *Acta Chirurgica Belgica*, or in Italy *Acta Phlebologica* — have devoted much space to history, through short historical reviews or dedicated sections.[1-4]

It is also worth mentioning that in 2015, the Academy of Fisiocritici, a learned society founded at Siena in 1691 and still active today, celebrated the bicentenary of the death of his past president Paolo Mascagni (1755-1815), one of the leading representatives of the eighteenth-century anatomy in Italy and, in particular, of the history of lymphatic system.[5-7] In 1787, Mascagni published a large folio volume, *Vasorum lymphaticorum corporis humani historia et ichnographia*[8], in which he described and represented for the first time, in 41 plates, the entire structure of human lymphatics by means of mercury injection.[i], [9] Mascagni's research on lymphatics was started in 1777 with experiments on bodies affected by extreme generalised oedema or dropsy: indeed, this particular diseased state, consisting of a massive swelling of the body, allowed him to quickly locate vascular vessels and, therefore, to trace the minutest ramifications of lymphatics throughout the body thanks to mercury injection. In 1784, the French *Académie des sciences* devoted a special competition on this topic, which, albeit quickly discontinued for lack of contributions, offered Mascagni the opportunity to outline a first explorative description of his anatomical experiences in a volume, *Prodrome d'un ouvrage sur le système des vaisseaux*

lymphatiques (1784) that is the basis for his further work.[ii], 10

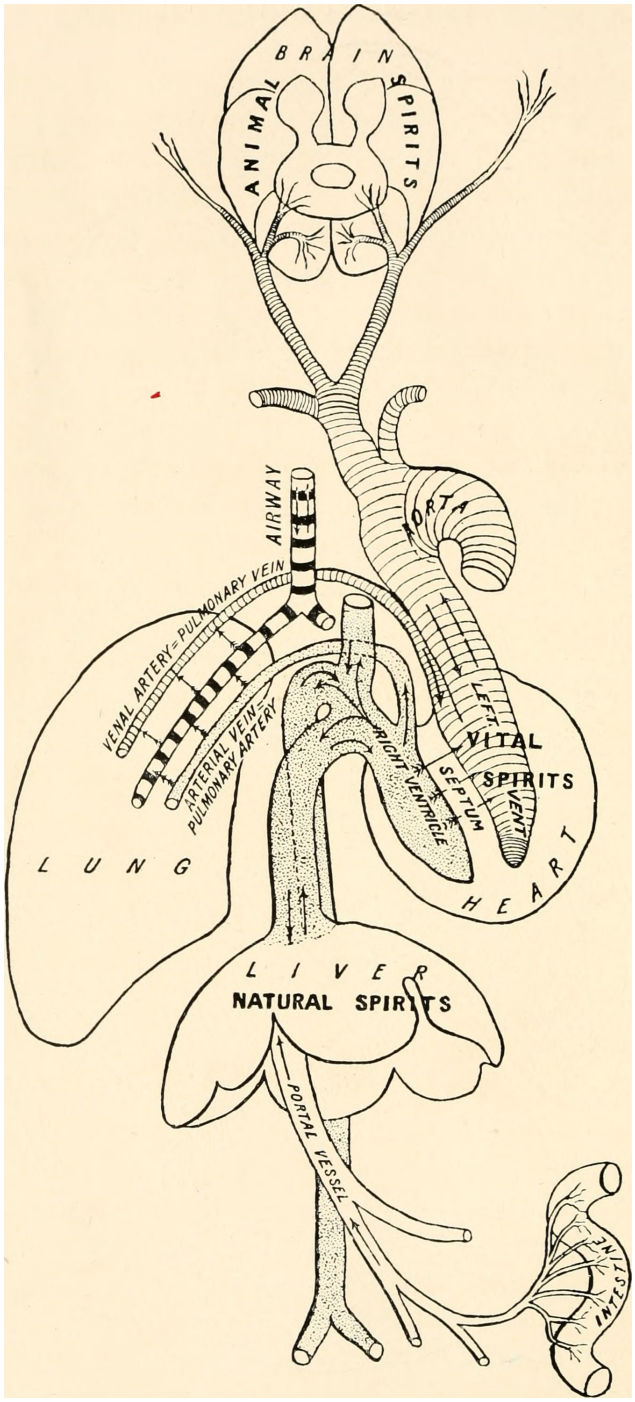

Figure 1 - Galen's vascular system. From Singer (1922, plate II).

The prolegomena to Historia et ichnographia shows Mascagni's accurate knowledge of the 16th and 17th-century secondary literature on lymphatics, as we can see in the most of the books that he personally owned, and that are now preserved in the library of the Academy in Siena[iii].

While recognising the pivotal role of Aselli's discovery of "milky veins," Mascagni himself highlighted the main consequence of Pecquet's work against Aselli's interpretation of lymphatics: disproving the haematopoietic function of the liver.

In this short paper, I will deal with exactly this issue, in order to understand how the early modern studies on lymphatics have considerably changed our conception of the human body. In particular, I will first describe the traditional view on this topic, which was influenced by Galen's conception of blood physiology; then, I will focus on two leading figures of the history of lymphatics: Gaspare Aselli and Jean Pecquet. I will analyse some scientific implications of their works for the modern conception of human body, the main of which is the refutation of hepatocentrism.

1. Liver's role in Galen's physiology and its legacy

Galen's vascular system distinguishes two different and separated paths of blood throughout the human body. In the first one, the blood, once produced by the liver, moves through the veins and reaches the upper and lower parts of the body, in order to feed them. In the second one, a part of the venous blood reaches the right ventricle of the heart by the vena cava inferior, then, passes to the left ventricle thanks to the meatuses of the interventricular septum allowing only the thinner parts of it to pass through. There, the blood, mixed with the *pneuma* coming from the lungs, is elaborated and pumped in the aorta, and finally reaches the other parts of the body through the arteries (see Fig. 1)[11].

This organisation of blood physiology implies that (1) Galenic vascular system is mainly "open-ended", because these two sub-systems, hepatic-venous and cardio-arterial, do not communicate, but they are completely independent of each other. Since it does not circulate, the blood cannot be recycled and is believed to be consumed throughout the body. Moreover, this organisation implies also that (2) there are two different kinds of blood: the blood flowing in the hepatic-venous sub-system is thick and raw because of the nutrients filled in, and it has a nutritive function. On the other hand, the blood flowing in the cardio-arterial sub-system is "vitalised" and it is aimed at bringing the pneuma throughout the body. Overall, according to Galen the liver plays a pivotal role in blood physiology, because it is the main source of the veins and it represents the body part, in which the process of haematopoiesis takes place.

In other words, the liver produces blood — in particular, venous blood — by transforming the chyle

received by the portal vein, just like a bakery that bakes and makes fit for nourishment the wheat collected in the storehouse. In Galen's analogy, the bakery is the liver, the wheat is the nutriment elaborated in the stomach, and the porters are the veins responsible for carrying the blood to the liver.[12]

Following the same analogy, Galen writes:

> When the liver has received the nutriment already prepared by its servants and having the crude outline, as it were, and indistinct semblance of blood, it provides the final elaboration itself so that the nutriment becomes actual blood. The impurities corresponding to the earth, stones, and seeds of wild plants in the wheat were eliminated from the food in the stomach, but this stands in need of a second cleansing from the impurities corresponding to the chaff and bran in wheat, and it is the liver which accomplishes this further purification of the nutriment (Ibidem, p. 205).

Thus, the liver represents the place for the second concoction that follows the first, preparatory elaboration happening in the stomach. However, how does this process occur? In *De usu partium* IV, Galen tries to identify which part of the liver is responsible for the origin of the veins and for the blood formation, and concludes that the only part to be considered is the flesh of the liver, i.e. its substance (Ibidem, p. 222). The process involved is that of assimilation: the nutriment is gradually reduced to the same substance of which the liver itself is composed — that is blood — by means of a complex network of veins that retains it long enough to be transformed:

> If a single, large cavity had been formed in the liver, the blood would not tarry there and only a very small portion of it would come in contact with the flesh of the viscus, with the result that sanguification would be impaired; for if the characteristic substance of the liver is the principal instrument of sanguification, nutriment that is more closely associated with this substance will take on the nature of blood more quickly and thoroughly (Ibidem, p. 226-7)

Finally, it is worth thinking about the fact that, for Galen, the flesh of the liver is the "main," but not the only part of the body responsible for the sanguification: in fact, it is supposed that also veins — precisely to the extent that they originate from the liver — could perform some mild form of haematopoiesis. This means that the meseraic veins carrying the chyle to the liver already make a change of some sort on the nutriment: interestingly, this specific point will be debated and refuted by Renaissance anatomists.

In fact, by arguing in favor of direct observation on human bodies (and not on animals), early modern anatomists reversed the relationship between authority and experience. As a result, the inaccuracies of Galen's view emerged and some of the main principles of his anatomy and physiology — such as the absence of pulmonary transit or the impermeability of the septum — were questioned. However, despite the evidence of Galen's errors, the centrality of the liver persisted. This is, even so, clearly exemplified by the way in which the study of human anatomy was still organized at that time: that is, according to the order imposed by Galen in *De anatomicis administrationibus* (osteology > muscles > arteries and veins > nerves > organs), paying particular attention to those who were considered the three main parts of the body — the liver, the heart, and the brain. Within a Galenic framework, the liver can only be considered the source of the venous system and the bodily part responsible for blood production.

This is the case of Vesalius who, despite having detected numerous mistakes in Galen, thus marking the birth of modern anatomy, failed to propose a valuable alternative to Galen's conception of the body[iv], [13], [14]. In the *Tabulae anatomicae sex* (1538)[15], his first anatomical contribution consisting of six different plates, he represented the vascular system according to the "old" anatomy, in accordance with Galen's authority.[16] Fig. 2 shows the plate I, which represents a five-lobed liver serving as "officina sanguificationis" with the entire ramification of the portal vein: in this way, the chyle is carried from the stomach and the intestines to the liver, and the waste product (the "succum melancholicum") excreted in the spleen. There is no difference with what was taught by Galen.

Plate II (Fig. 3) is instead devoted completely to the description of vena cava, by which blood can reach all the parts of the body and feed them ("qua sanguis omnium partium nutrimentum per universum corpum diffunditur").

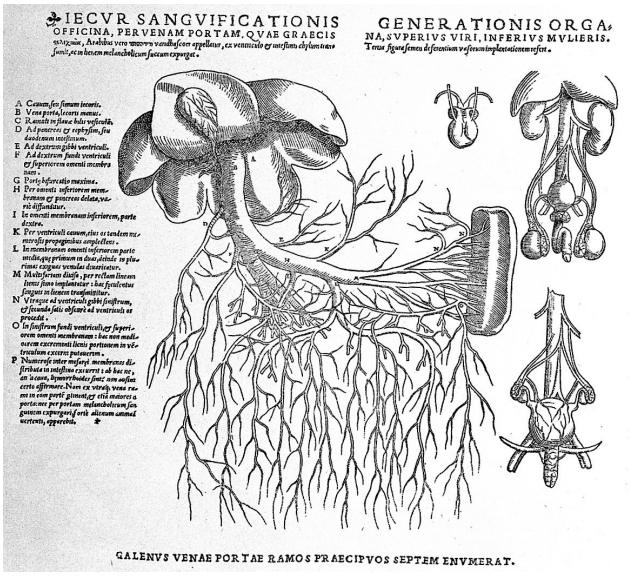

Figure 2 - Plate I: Description of the liver, the portal system and the organs of generation (male/female). From: Tabulae Anatomicae sex (1538)[15].

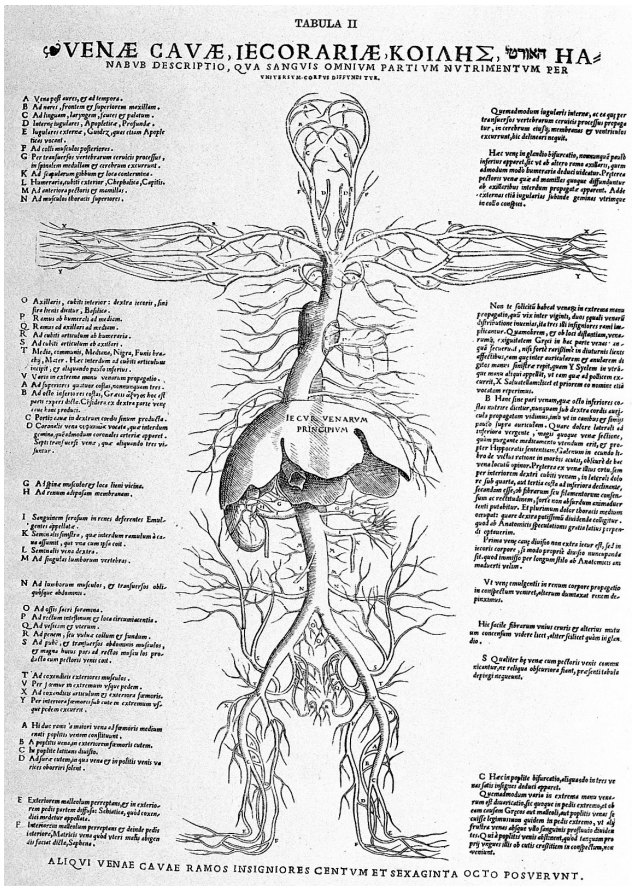

Figure 3 - Plate II: Description of the caval system. Liver is labelled: "Iecur venarum principium" (Liver source of the veins) (Vesalius 1538, Plate II).

Figure 4 - Description of the portal system. From Vesalius (1543).

At that time, Vesalius still believed — in respect (again) of Galen's authority — that the entire venous system had originated from the liver (here labelled as "iecur venarum principium"), including caval system, which is depicted as a continuous vessel.

Vesalius revised this position in his masterpiece *De humani corporis fabrica* (1543)[v], [17], [18]. First of all, he rejects that representation of the liver in five lobes that he probably borrowed from medieval anatomists. Nevertheless, this idea implicitly recurs in the fact that, anyway, the portal vein entering the liver is supposed to be branched in (again) five different vessels (see the top of fig. 4) (Singer 1946, p. lviii-lix)

Secondly, contrary to what previously believed, he abandons the argument advanced by Galen about the origin of the caval system and rather maintains that the vena cava arises directly from the heart, without necessarily denying the flow of blood from the liver, or at least from the offshoots of the portal vein.

Finally, he revises the process of bloodmaking: although still attributing it to the liver, Vesalius does not recognise a form of haematopoiesis, albeit weak, to the portal veins:

> The most and juicy product of the processing carried out by the stomach is gathered in from the intestines and from the stomach itself by the branches of the portal vein and conveyed to the liver. I would not agree that these branches, before conveying the juice or pulp to the liver, make it into a crude form of blood and so, as Galen claims, prepare it in exactly the same way as the liver does; for if the veins (and not only these ones but all offshoots of the hollow vein) had, in addition to the power of transforming their own proper nutriment, a power of transformation like that in the stomach serving the body as a whole, they would change the contents of the veins, not into something red, but into something white: the rule is that in nutrition the thing transformed takes the color of the thing transforming. The liver takes into itself and into all the branches of the portal vein that spread through its substance all the moisture and juice processed by the stomach and, by virtue of the transforming power innate in its own substance, makes it resemble itself as closely as possible, thus generating blood.[19]

Liver's pivotal role is supported also by Realdo Colombo in his main work *De re anatomica* (1559), published a few

years later the second edition of *Fabrica* (1555)[20-21]: aside from denouncing the errors of Galen, Colombo critically reflects also on the work of Vesalius. On the one hand, in accordance with Galen, he claims that the liver is the "omnium venarum caput, fons, origo et radix,"(Colombo 1559, p. 164) whose task is making blood. Thus, as a vein, the portal vein arises from the concave part of the liver. Then, its main trunk is divided in seven branches. The first two of them — which we can compare with what today we call the left and right gastric veins — reach the upper and lower part of the stomach, respectively. They both have the task of feeding the stomach. The third branch reaches the omentum. The fourth, as a sort of splenic vein, conveys the black bile humour to the spleen. The last three branches, the fifth, the sixth, and seventh, all serve the intestine, but at different points: correspondingly, the colon, the small intestine, and the rectum (Ibidem, p. 164-165). At this point, Colombo makes a very often neglected remark: he claims that the innumerable and extremely small veins in which are divided the branches reaching the intestine, i.e. the meseraic veins, have valves at their ends. Just as what happens in ureters, valves prevent the chyle from turning back. In other words, they give it a specific direction, from the intestine to the liver, which is in charge of transforming it into blood. In this way, accidentally, Colombo not only identifies the chyliferous vessels (meseraic veins), but, by highlighting the existence of valves inside them, he somehow anticipates the idea of a flow of chyle, that anyway cannot be defined circulation yet (Ibidem, p. 165; Colombo 2014, p. 456, n 12). On the other hand, contrary to what claimed by Galen in *De usu partium* IV, Colombo, just like Vesalius, denies to the portal vein and its ramifications a power of sort of transforming the chyle into blood.

Unfortunately, the lack of anatomical plates has severely limited the dissemination of Colombo's work compared with that of Vesalius, although Colombo's attacks on Galen's physiology are more explicit. Colombo, for example, strongly denied the existence of the meatuses of the interventricular septum, arguing that the passage of blood from the right ventricle to the left one was possible only by assuming the existence of a pulmonary transit (Colombo 1555, p. 177).

2. From Aselli to Pecquet: A challenge to Galen's physiology

The most common 17th-century anatomical treatises continue to emphasize the pivotal role of the liver. It is thus hardly surprising that Aselli too had worked in a Galenic framework. Gaspare Aselli (1580-1625) was professor of anatomy at the University of Pavia in 1624-25[22] (Fig. 5)[23]. His main work *De lactibus sive lacteis venis*, based on his four lectures on lacteals held in 1625, was published posthumously in 1627 by his friends and colleagues Alessandro Tadino and Senatore Settala, only one year before the publication of William Harvey's work on blood circulation (*De motu cordis*)[vi], [24]. This book consists of thirty-five short chapters, provided with four folio-size chiaroscuro woodcuts of canine lacteal vessels, that probably represent the first colour-printed anatomical illustrations[25].

Aselli aims here to show the existence of a new kind of vessels, the chyliferous vessels, which he names "milky veins". The discovery of what seems to be a fourth vessel (the other three being the arteries, the veins, and the nerves) is the result of a dissection on dog occurred in 1622. At the bottom of the beautiful title page engraved by Cesare Bassano, there is a clear reference to the chyliferous vessels and their insertion in the liver, which is the main topic of the work (see Fig. 6).

It is firstly worth noting that Aselli's work follows Fabricius' format for anatomical reports, that consists of a three-section structure: *historia, actio, utilitas*. Anatomists should firstly provide a full description of the concerned body part, without any tentatively reference to causes. This means that the first section, called "historia," focuses only on the structure of the part. Secondly, anatomists should proceed with "actio," that is the description of the action of the part. Finally, the last section, "utilitas," should offer the account of the final causes, that is, the reasons why that part exists[vii], [26-28].

In the section devoted to *historia*, after describing the main mesenteric vessels, Aselli provides a detailed report of his accidental discovery ("De Quarto, & novo Vasorum Mesairacorum genere"). On July 23, 1622, Aselli dissected a post-prandial dog, in order to see the recurrent nerves and the movements of the diaphragm. However, the opening of the abdomen and the displacement of the intestines suddenly revealed something completely new, that is "a great number of slender cords, so to speak, extremely thin and white, scattered all over the whole mesentery and intestine, from almost innumerable starting points"[viii], [29]. Aselli believed those "cords" to be nerves, but then he realized that this hypothesis was untenable. Influenced by the scholarly disputes on meseraic veins, he decided to lance one of those whitish filaments so as to look at it inside. He noticed a milky liquid filling in, a feature that suggested him the name of "venae albae et lactae." Then, in support of his discovery, he dissected another dog, but without finding the new vessels. But, since this second dog was unfed, he inferred that there was a correspondence between the food intake and the chance to detect the chyliferous vessels. Thus, he tried again with a third dog, but after six hours from the animal's last meal. Indeed, the vessels reappeared.

Figure 5 - Gaspare Aselli's portrait, by Cesare Bassano.

Figure 6 - Engraved title page, by Cesare Bassano. At the bottom: Anatomical illustration of the chyliferous vessels, labelled with the Latin name "Lacteae venae." From: De lactibus sive lacteis venis (1627).

Nevertheless, despite the novelty of his discovery, Aselli continues to support Galen's physiology: as a consequence, chyliferous vessels are considered to lead to the liver, which inevitably preserves its privileged position as the organ of sanguification. "Le mensonge se mêle ici à la verité"[30], as Portal wrote in his *Histoire de l'anatomie et de la chirurgie*. In particular, by describing the *progressus* of chyliferous vessels, Aselli confused a large mesenteric lymphatic gland with a pancreas of sorts (now known as Aselli's pancreas or Aselli's gland), from which larger vessels were supposed to reach the caval vein, the portal vein, or directly the liver (Ibidem). In Fig. 7, all milky veins scattered in the mesentery (marked by the letter B) converge in Aselli's pancreas (letter L).

Evidently, the idea of chyle flow which follows from this explanation of the action and the use of chyliferous vessels prevented Aselli from correctly finding the thoracic duct, which is the necessary step to make Galenic view's refutation possible. In fact, it is necessary to show that the chyle cannot reach the liver.

Numerous historians of medicine agree to highlight the contraposition in Aselli's work between personal experiences by means of direct observations and dissections and the provided theoretical explanations. In this regard, for example, Portal said that "L'Auteur se perd dans une théorie qui n'est appuyée sur aucun principe de physique" (Ibidem): Aselli would not have supported Galen's theory, if he had been aware of the existence of the thoracic duct, which was already observed by Eustachi in horses. "En adaptant la découverte de ce grand homme à la sienne," he wrote, "Asellius eût conduit le chyle dans la veine souclaviere gauche, & non dans le foie" (Ibidem). Similarly, in 19th century, Charles Daremberg said: "Encore une fois, voilà ce que produit la mauvaise physiologie ou la physiologie *a priori*, la physiologie non expérimentale. Le hasard fait trouver les chylifères, la théorie galénique fait qu'on les voit se terminer au foie"[31].

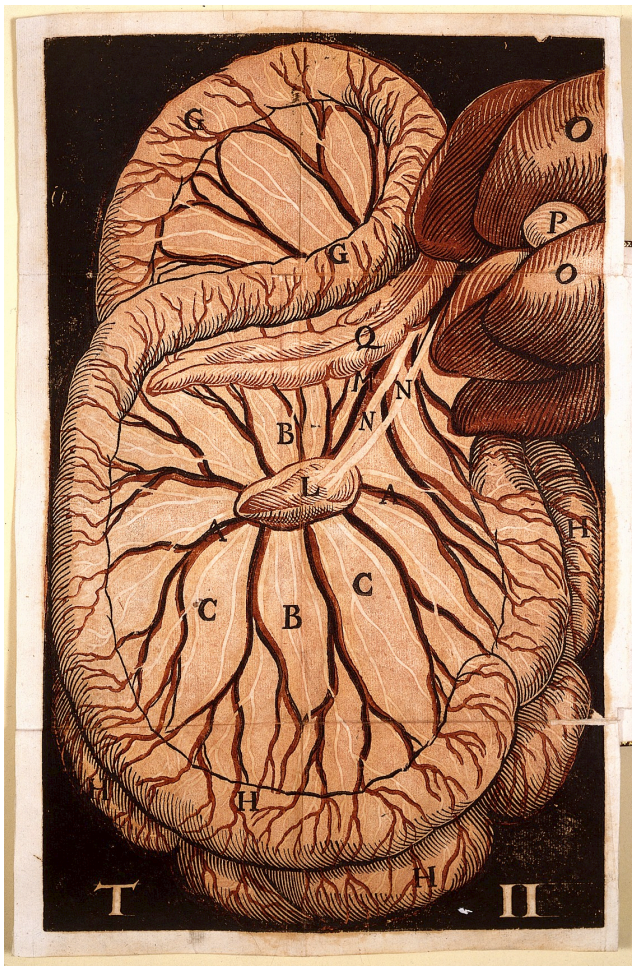

Figure 7 - One of the four chiaroscuro woodcuts. This plate, in particular, shows the relationship between the mesentery and the liver by means of the so-called "Aselli's pancreas." From: De lactibus sive lacteis venis (1627).

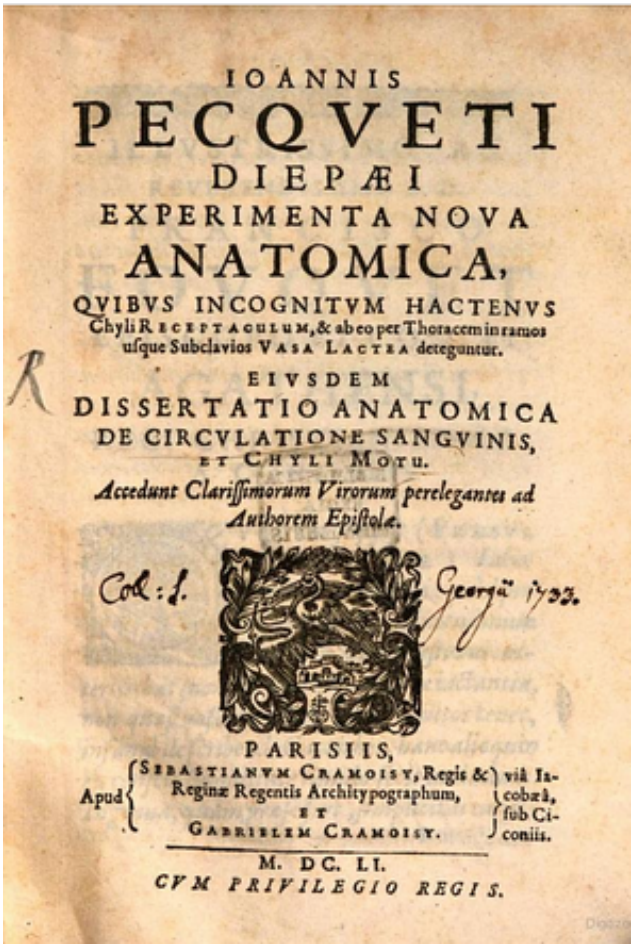
IOANNIS
PECQVETI
DIEPÆI
EXPERIMENTA NOVA
ANATOMICA,
QVIBVS INCOGNITVM HACTENVS
Chyli RECEPTACVLVM, & ab eo per Thoracem in ramos
usque Subclavios VASA LACTEA deteguntur.
EIVSDEM
DISSERTATIO ANATOMICA
DE CIRCVLATIONE SANGVINIS,
ET CHYLI MOTU.
Accedunt Clarissimorum Virorum perelegantes ad Authorem Epistolæ.
Col: I. Georgii 1733.
PARISIIS,
Apud SEBASTIANVM CRAMOISY, Regis & Reginæ Regentis Architypographum, ET GABRIELEM CRAMOISY. via Iacobæa, sub Ciconiis.
M. DC. LI.
CVM PRIVILEGIO REGIS.

Figure 8 - Title page of Jean Pecquet's Experimenta nova anatomica (1651).

Jean Pecquet's *Experimenta nova anatomica* (Fig. 8)[32], published in 1651, can be viewed as a turning point in the studies on the lymphatic system, because it proves the existence of new anatomical structures involved, such as the 'receptaculum chyli' and the thoracic duct, and finally shows the chyle's correct path[ix], 33, 34. This discovery, described in only about twenty pages by means of a single anatomical table, actually has important implications for the early modern medicine, as I will explain.

As he says at the beginning of his work, Pecquet does not dispute Aselli's discovery of milky veins, but he refuses his view on the chyle as it is still too dependent on Galen's hepatocentrism. Aselli has mistakenly taken for granted and not really verified that the milky veins gather in the liver's fissure. Numerous anatomists have debated on this issue (Walleus, Harvey, Conring, Bartholin, and Riolan too), arguing that a part of the milky vessels, scattered in the mesentery, meet in the pancreas, a part of them in the liver, a part of them in the vena cava, yet another part of them in the portal vein: Pecquet wants to demonstrate that the chyle is not collected by any of these parts.

Thus, a series of vivisections on a variety of animals have been performed for about three years, starting from 1647. These direct experiences on live animals allowed Pecquet to discover that, contrary to what was previously supposed, the chyle flows from the intestines into a sort of dilated sac, named the "receptaculum chyli." Then, it is led to the thoracic duct, a ladder-like structure through which it reaches the subclavian vein so as to finally meet the blood in the heart (Fig. 9). This implies that the liver is deprived of its traditional function because anatomical

dissections demonstrate that it cannot receive the blood from the intestines. Then, what is its new role? Liver mainly serves as a means to "catalyse" digestion and filter the blood:

> Praeter eam, qua fungitur, ut supra dictum est, vicem pistilli subjectas in infimo Ventre partes, Respirationis motu, percutientis, Hepar ingenti, quod accipit à Porta, Sanguinis profluvio calorem in ciborum elixationem Ventriculo subministrat; & Sanguinem ipsum idoneo transcolat Parenchymate, utque Renes eundem sero repurgant, & Lien aciditate Vendicat, sic Hepar admixtae Bilis expedit consortio (Pecquet, 1651, p. 86).

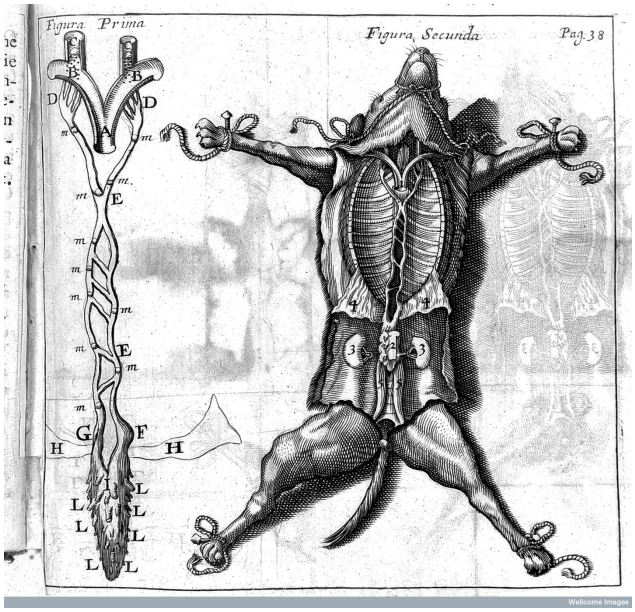

Figure 9 - Pecquet's anatomical illustration of a dissected dog. The thoracic duct is represented in two different ways: magnified (on the left) and in its natural proportions (on the right). From Pecquet (1651).

Moreover, in order to prove the relationship between chyle flow and blood circulation, Pecquet tries to produce not only new evidences in support of Harvey's theory, but also a valuable causal explanation of blood motion (and, consequently, of chyle), without any reference to Galenic faculties. He believes that air's elasticity, that he infers from 1640s experiments on Torricellian tube, can *mechanistically* explain blood and chyle motion[x], 35, 36.

3. Some clinical and methodological implications

Pecquet's work has decisively influenced the modern conception of the body. We can draw some important anatomical, clinical, and methodological implications:

- 1) Through the demonstration of the thoracic duct, Pecquet launched one of the strongest attack on Galenism: since the blood does not pour out in the liver, the liver cannot exert the process of concoction, by transforming the chyle into blood. As a consequence, the liver lost its privileged role in the body.
- 2) Moreover, Pecquet proved that the chyle flow is circulatory. In 1653, in *Vasa lymphatica*, the Danish anatomist and physician Thomas Bartholin supported Pecquet's conclusions and showed that chyliferous vessels belong to a new vascular system, named "lymphatic system."
- 3) These discoveries gradually made it possible also to reveal new lymph borne diseases, as already suggested, albeit partially, by Aselli in the last chapter of his work (Aselli 1627, ch. 35)[37].
- 4) Finally, Pecquet's three-year experiments on live animals pose the problem of evaluating the impact of vivisections on life sciences[38]. Moreover, the attempt to adapt physical researches to medicine shows the importance of collaborations between physicians and mathematicians in the foundation of a "mechanistic anatomy" and, more generally, in the development of late-17th-century medicine.

Endnotes

[i] For recent studies on the use of mercury in medicine and chemistry, see Hendriksen (2014).

[ii] The history of his experiments on lymphatics are outlined in the preface.

[iii] See, for example, all the contributions in the book section "Passioni di scienziato: I libri della biblioteca di Paolo Mascagni" (Vannozzi 1996).

[iv] The amount of secondary literature on Vesalius is huge. For an updated and annotated bibliography, see "Vesaliana," compiled by Maurits Biesbrouck and available from http://www.andreasvesalius.be. On Vesalius's anatomy, see Carlino (1994). See also Vons (2014).

[v] A second edition was published in 1555. Notes for a supposed third edition are analysed in Nutton (2012).

[vi] Aselli's unpublished manuscripts are described in Ducceschi (1922). Aselli's four lessons on lacteals ("Lectiones de venis lacteis - Gasparis Aselii - de anno MDCXXV Ticini") are preserved in the Archive of the Civic Museum of Pavia.

[vii] On Fabricius's three-section structure, see Cunningham (1985, 1997) and Siraisi (2004).

[viii] The description of Aselli's discovery is outlined in Aselli (1627, ch. 9). A partial English translation of this passage is provided by Pomata (2005).

[ix) On Pecquet, see Bertonoli Meli (2011) and Guerrini (2015).

[x] Part of these experiments are reported and discussed in the dedicated section "Experimenta physico-mathematica de vacuo." Unfortunately, due to space limitation, a full account of Pecquet's

arguments is omitted here. More details in Webster (1965) and Bertoloni Meli (2008).

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COMPRESSION ORIGINAL ARTICLE

A new hybrid protocol enabling to evaluate the pressure level of medical compression stocking in patients: "ex-vivo procedures".

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Abstract *Introduction* Medical compression treatment using stockings exerts a pressure to the vascular system which represents the therapeutic dose. This pressure is indirectly measured according to heavy laboratory tests based on calibrated artificial leg markings. The objectives of this work were to elaborate and test a new dynamometer which allows direct measurement of pressure from how the stocking is applied by the patient and whatever the leg curvature radius are.
Method A portable mini dynamometer (Modyn) that reproduces the testing in textile laboratories was elaborated. To test the reliability of this device four sizes, each size available in three lengths, of anti-thromboembolic medical compression stockings were measured at the ankle level using both the Modyn and the reference textile laboratory dynamometer. All the measurements were blindly performed. The measurement procedure followed the G 30 102 B French norm.
Results On a total of 72 measurements, the explained variance between both methods was r^2= 0.92 and the correlation coefficient r= 0.96. The intra-operator repeatability of the Modyn provided a mean value of 22.12 mmHg, SD 0.74.
Conclusion This portable, easy to use, pressure measurement device using the dynamometer principle, is a reliable field tool that should be used in future clinical trials to measure more precisely what the patients treated by compression stockings really receive as therapeutic pressure.

Keywords Medical compression stockings, Pressure measurement, Dynamometer, Interface pressure, Lower limb

Introduction

Compression therapy is a leading treatment of superficial venous disorders and can be applied to patients by using bandages, medical compression stockings (MCS) and sustained or intermittent air pressure therapy. Bandages are more frequently used as a first line treatment in severe cases such as ulcers. Notwithstanding the efficacy, the applied pressure to the diseased leg is mainly linked to the operator's skills due to the Laplace's law type of pressure. The pressure profile applied by air pressure therapy devices, accurately controlled by the machine, is based on the Boyle-Mariotte's law: PV = MkT (P = pressure, V = volume, T = temperature and M = mass, K = coefficient)[1], which is the principle of the arterial pressure cuffs. In spite of the benefits in pressure level accuracy, air pressure therapy is by far less ambulatory than bandages or stockings. MCS, being ambulatory, are ideally supposed to have a controlled pressure profile on the patient's legs.

MCS are classified in four compression classes, corresponding to different therapeutic dosages. They are prescribed to patients according to their degree of pathologies[2]. Numerous national and international recommendations for prescriptions have been issued by medical societies. One of them, widely considered as the most relevant statement on compression therapy, highlights the limits of our knowledges on the compression treatment[3]. In many countries, MCS are certified through a normalized process. In France, independent technical laboratories use the French norms AFNOR G30.102B to evaluate the MCS pressure[4]. This procedure, called "in-vitro" procedure, firstly requires to don and mark the MCS on a normalized Hoheinstein wooden leg (with dimensions corresponding to the MCS size) and secondly to place the MCS on a standard and heavy dynamometer to stretch the MCS, simulating the leg dimensions[5]. The results will indicate the amount of pressure exerted at specific points such as the ankle, the calf and the thigh.

In spite of its perfect adequacy with the industrial process for MCS manufacturing, that procedure is not satisfactory for clinical purposes. Indeed, in spite of the almost perfect characteristics of the MCS, following rigorous qualification and certification systems based on the national standards of many countries, the question of the effective pressure, as really applied to human legs, is still pending.

To better evaluate the true pressure applied to the patient's legs, numerous protocols labelled "in-vivo" protocols have been developed[6]. Those cheap, fast and easy-to handle devices and procedures consisted in placing a small pressure sensor between the skin and the MCS fabric.

Unfortunately, the results depended mostly on the exact location of the sensor on the leg (local limb curvature) and less on the apparatus accuracy. This protocol is known as highly operator dependent[7]. Subsequently, it is almost impossible to compare the pressure level mentioned on the packaging and being the official characteristics of the MCS, and the pressure level measured using the "in-vivo" procedure.

Moreover when for instance, the calf muscle contracts, the curvature (local limb radius), varies in such a proportion (values at certain points of the leg) that the "in-vivo" technique would rather measure the muscle anatomical properties than the intrinsic MCS properties themselves[8]. Some authors call that the "working-resting pressure". Therefore, "in-vivo" and "in-vitro" measurements can be compared only when "in-vivo" sensors are placed on a human leg on a very precise place which has a curvature value corresponding to the circumference of the wooden leg used for the "in-vitro" testing[9].

For example, to measure the "in-vivo" pressure on an ankle of a 23 cm circumference the sensor must be placed on an area where curvature radius is of 3.67 cm. In addition, the investigator must be equipped with a device enabling the measurement of the local limb radius. In order to address these questions, a new procedure is being elaborated and a new device is designed aiming at providing "in-vivo" results which would be comparable to the normalized "in-vitro" figures. We name this technique "ex-vivo" protocol.

Method

Instrumentation / Tool.This new device is a portable mini-dynamometer that reproduces the "in-vitro" testing usually performed by using a usual (non-portable) dynamometer in textile laboratories. It is a non-destructive testing method since the patients can get back to their stockings after the "ex-vivo" procedure is completed. The specifications of this device are provided in Figure 1. We call it "Modyn", an abbreviation of Mobile Dynamometer. In a nutshell, this hybrid "ex-vivo" method allows the investigator to obtain figures of pressure close to the true effective pressure independently without the curvature radius value.

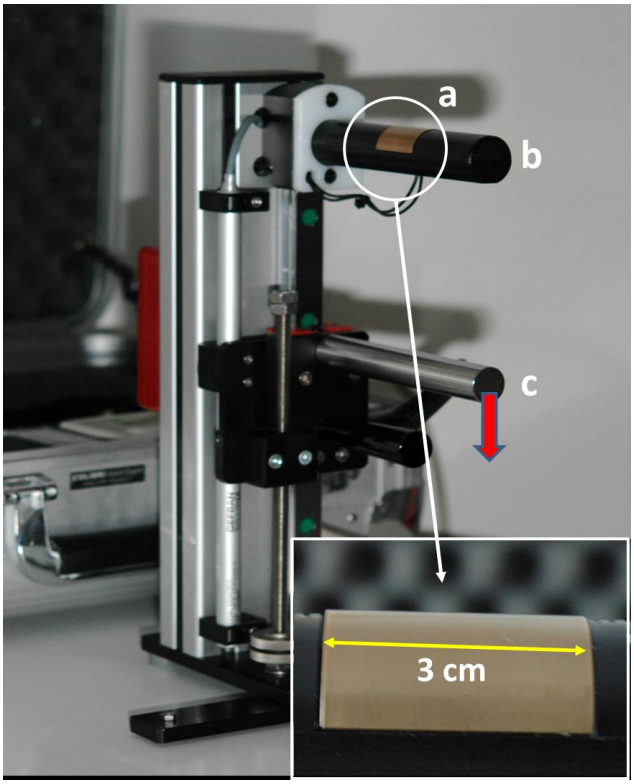


Figure 1 - The MObile DYNamometer (MODYN) device. a) the force sensor, b) the fixed part and c) the mobile part. Enlarged, the force detection zone of 3 cm length.

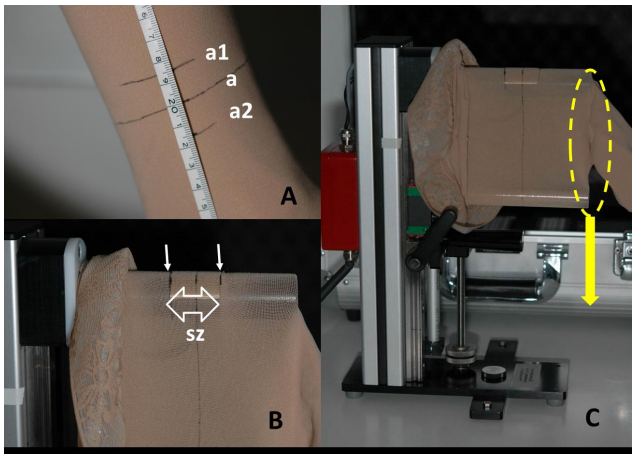


Figure 2 - Principle of ex-vivo measurements. A. Marks tracing on MCS when worn on leg; a, circumference line, a1, above mark at 1.5 cm and a2 below mark at 1.5 cm from the circumference line. B. Placement of marked MCS on Modyn and positioning the marks (white arrows) on the sensor zone (sz). C. The fabric is stretched at the related circumference using the mobile part of the Modyn (yellow arrow).

The methodology for the pressure measurement is performed in four steps.

1) firstly, the patient puts the MCS on his or her leg as he or she is usually doing without any interference/training from the investigator.

2) secondly, the investigator measures the limb circumference at the point where he wants to know the amount of pressure exerted by the MCS and traces a line around the limb at that point. At an equal distance of 1.5 cm from this circumference line, two marks are proximally and distally traced on the stockings. Those 3 cm (distance between the 2 marks) correspond exactly to the sensor zone of the Modyn (Figure 2).

3) thirdly, the patient wears off the stocking. After what, the stocking is positioned on the Modyn. A gentle massage is performed in order to homogenously distribute the MCS fabric; the 2 marks performed at the second step must be placed at the edges of the force sensor of the Modyn.

4) finally, the MCS is stretched at an elongation corresponding to the defined circumference value at the second step. The obtained results are compared to the data issued from the measurements performed on a usual dynamometer in a textile technical laboratory using the equipment required by the NF G30-102B norms.

Protocol

The objective of the present task is to compare the data obtained from the Modyn to the data issued from the Usual Dynamometer in textile laboratory (UD) which is cited in the NF G30-102B norm. The same MCS are involved; they are tested at the same marked areas and stretched within the same circumference. Only the attachment of the MCS fabric to the force sensor differs from Modyn to UD. The tested stockings were anti-thromboembolic medical compression stockings (ATE) manufactured by SIGVARIS (Saint-Just-Saint-Rambert, France). Four sizes, T1 (size 1) to T4 (size 2), of this same product range were involved; each size is available in three lengths (which are short, normal and long. Both items of each pair were tested. In total twenty four MCS were evaluated and twenty four measurements collected.

The stockings were donned on the leg of one volunteer having a 22 cm ankle circumference. The evaluation process followed the procedure detailed in the previous paragraph. Additionally, the range of measurements was extended to two other dimensions, 21 cm and 23 cm so as to consolidate the validity of the Modyn. In total seventy two measurements from Modyn were compared to seventy two measurements from UD.

Modyn measurements were carried out by a qualified investigator having adequate skills regarding the device. Then, the stockings were sent to a textile laboratory in order to be measured on a UD; they were carried out by a technician well-trained on the UD measurement techniques. All the measurements were blindly performed; the operators didn't know the size nor the compression class of the MCS. They were only requested to test the fabric at the marked area which was the ankle zone, by stretching them at 21cm, 22cm and 23 cm circumferences.

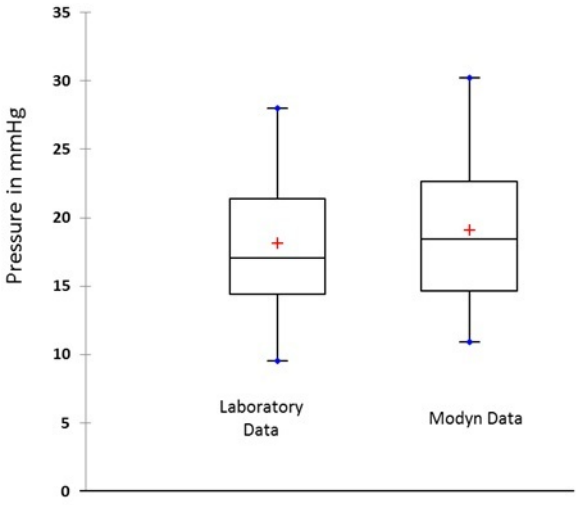
Statistics

The descriptions used basic statistical parameters: mean and standard deviation for quantitative variables, and frequency and percentage for qualitative variables. To build a relationship between the two quantitative variables, the statistic correlation was used and the Pearson linear correlation coefficient (r) calculated.

Results

If we consider only the "ex-vivo" results which represent twenty four measurements on all sizes and lengths, stretched to 22cm related to the involved patient having 22cm ankle circumference, the mean pressure obtained on Modyn was 19 mmHg, SD 5.52 and the mean pressure obtained by UD was 18 mmHg, SD 5.52 (Graph 1). The explained variance between both methods was r^2= 0.93 and the correlation coefficient r= 0.96 (Graph 2).

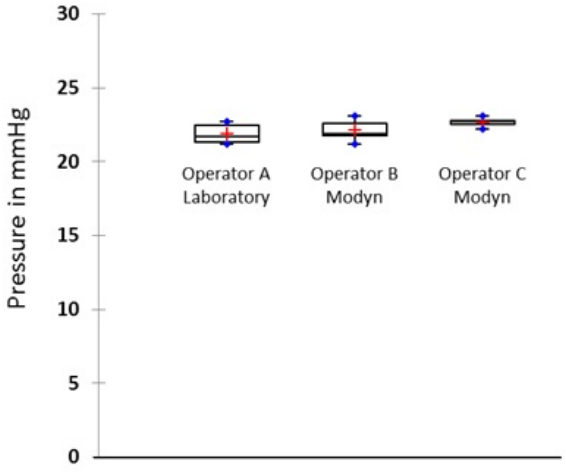
If we compare the overall seventy two figures (all items tested at 21cm, 22 cm and 23 cm), the explained variance was r^2=0.92 and the correlation coefficient r=0.96 (Graph 3).



Graph 1 - Comparison of pressure values laboratory dynamometer (left) versus Mobile dynamometer (right) on 24 measurements of all sizes at 22 cm of ankle circumference. Mean, SD, 5 and 95 percentiles.

The intra-operator repeatability of the UD within five measurements carried out on same above item (size 2N stretched at 22 cm ankle circumference) provided a mean value of 21.9 mmHg, SD 0.68. The intra-operator variability of the Modyn within five measurements carried out on size 2N stretched 22 cm ankle circumference provided a mean value of 22.12 mmHg, SD 0.74.

Last but not least, another set of five measurements performed by a third operator on Modyn carried out on the item mentioned above (size 2N stretched 22 cm ankle circumference) provided a mean value of 22.6 mmHg, SD 0.34. Therefore, the variability inter operator on Modyn is minor.



Graph 2 - Intra operator repeatability of measurements on Modyn. Mean, SD, 5 and 95 percentiles.

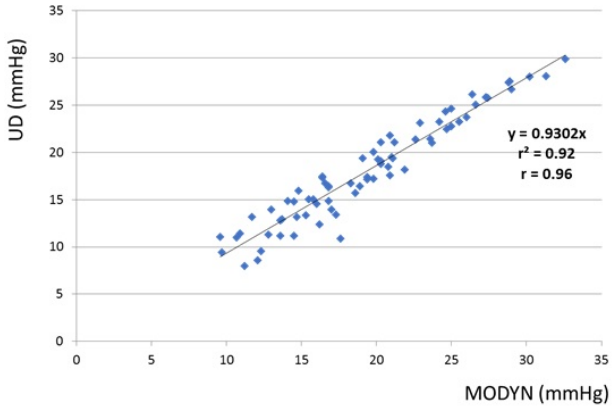
Discussion

Medical compression treatment involving stockings (MCS) is based on the application of an interface pressure above the skin and all along the lower limb; the pressure being partially transmitted to the venous system. Its role, in superficial and/or deep venous insufficiencies, is to counterbalance the intravascular venous hypertension caused by venous disorders. As a consequence, the transmural pressure equilibrium is reduced to a more normal level. The MCS effect is then a question of pressure amount.

MCS are ranged in four classes according to the pressure exerted on the ankle. That supposes that the ankle circumference of the patient who will wear a given MCS, would correspond to the size. That also underlines the importance of the marking process of the area to be stretched by the dynamometer. The norms recommend the marking task to be performed on wooden Hohenstein standard leg or Marking board.

Unfortunately, very little is known about the pressure effectively applied to patients in published trials on compression therapy. We only know the theoretical mean pressure exerted by the stocking used in the trial, but nothing beyond that. Besides the true morphological status of the patient's leg with pressure exerted along his or her leg, and not only at the ankle level, are not precisely known.

The new procedure described here-after permits to fill this gap and provides a field device to measure the pressure on a given patient's leg in the clinical trials. According to our new procedure, the marking process is performed directly on the patient's leg when the MCS is already worn. By consequence, the process will measure how the patient, once the MCS put on, had spread the threads along his or her leg.. The "ex-vivo" procedure allows to evaluate the effective pressure exerted by a given MCS on a given patient's leg, donned in a given way.



Graph 3 - Overall correlation of pressure measurements, UD (Usual Dynamometer) versus MODYN (MObile DYNamometer).

Consequently, it provides the closest value to the true and effective pressure exerted by the stocking on the patient's leg. That is the innovative part of the "ex-vivo" method. The results demonstrate that the Modyn device is not only a practical device but is also a reliable tool as its results are perfectly correlated to the data issued from the currently used method in textile laboratories (UD) which complies with national norms.

According to the results mentioned above, it would be interesting to use this "ex-vivo" procedure with mobile dynamometer or usual laboratory dynamometer, in clinical studies involving MCS. It would allow us to know the effective interface pressure, in other words, the dosage of the compression treatment without any interference from the local limb radius.

Nevertheless, it must be highlighted that the main parameter causing the interface pressure variability is more related to the donning action, namely how the patient stretches the stocking on his leg, than the performance of the instrument in use. The adequacy of the MCS size with the patients' leg dimensions is also less important than we thought.

Conclusion

The Modyn, which is a portable- easy to use- pressure measurement device using the dynamometer principle, is a reliable field tool that should be used in future clinical trials to measure more precisely what the patients really receive as therapeutic pressure.

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IRON PATHOPHYSIOLOGY REVIEW

The role of free iron in cardiovascular diseases. Part II

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Abstract This work is the second part of a review on the role of free iron forms in cardiovascular diseases. Here, we stress on various aspects related to the iron form that are free of poorly chelated to iron biocarriers, as for example the NTBI (*Non Transferrin Bound Iron*). We report on problems relating the determination of such free iron forms and, after all, on their implications in cardiac diseases as well as in venous insufficiency.

Keywords Free and poorly chelated iron; Biocarriers; Cardiovascular diseases; Venous insufficiency

Non-Transferrin Bound Iron

In the first part[1] of the review we outlined the role of free iron in vascular diseases. Actually, it is well known that most of the body's iron is bound to the protein and non-protein biomolecules (carriers) in order to render it bioavailable, so as to move it between the various compartments and overcoming the great toxicity that characterize the free iron, due to its ability to create organic bounds, generating oxidative and nitro-oxidative stress. The most relevant iron carrier is the transferrin, while the main iron deposit is the ferritin. In recent years great attention has been paied to the non-transferrin bound iron (NTBI), a form of free iron highly reactive. Such poorly chelated form of iron could play a role in a number of pathologies and cannot depend on a systemic overload condition of iron. This should lead to a reflection since, for example, even in a condition of iron-deficient anaemia it is possible to find such toxic forms of iron released by their transferrin and ferritin carriers.

The non-transferrin bound iron (NTBI) forms, also denote as non-protein bound iron (NPBI), are grouped in different chemical forms with different characteristics. The cytoplasmic labile iron pool (LIP) is involved in the regulation of the cytosolic iron regulatory proteins (IRP)[2]. Further evidences show that there exist unbound forms also for the copper (non-ceruloplasmin bound copper, NCBC), that are toxic and relevant in the pathogenesis of neurodegenerative[3,4] and cardiovascular diseases as well as in the atherogenic process[5]. The term "*Labile Iron Plasma, LIP*" was coined by Greenberg and Wintrobe in 1946[6]. It was then modified by Jacob in 1977 in "*Transient Iron Pool, TIP*"[7], while Kruszewski[8] refers to it as "*Labile Iron Pool, LIP*", that is, as the poorly chelated iron with low molecular weight that is able to rapidly cross the cells. Some authors[8,9] have also used the term of "*catalytic iron pool*" to indicate these scarcely bound, highly reactive iron forms, able to set off oxidative stress. Chelation kinetics studies have shown these "*iron free*" forms starting from the chelators used and, in this way, a number of techniques have been describes, such as:

- BDI: bleomycin detectable iron[10]

- DCI: desferrioxamina chelable iron, a term used by Breuer to indicate NTBI iron[11]
- BPS: bathophenantroline-disulphonate, a chromogenic chelator detecting the free iron at 532 nm[12]

In normal conditions, the transferrin is saturated with the iron about at 20-35% (20 µmol/L), and this represents the main non-Heme circulating iron form. However, the transferrin saturation can increase, ensuring in this way an effective chelating mechanism and neutralizing a possible increase in the iron amount. For this reason, in a healthy individual the circulating NTBI (*Non Transferrin Bound Iron*) is essentially absent, its plasmatic levels being less than 1 mol/L, hard to detect by the majority of actual lab methods[13]. In pathologic conditions due to iron overload, such as thalassemia, hemochromatosis etc.[14,15], a progressive increase in the free NTBI iron content also corresponds to an increased iron saturation of transferrin. While this mechanism is intuitive and explicable[16] its contrary, that is, normal or little increased levels of transferrinic saturation and increased circulating free iron, is less easy to understand[17].

For this reason the early studies on NTBI iron were mainly performed in iron overload conditions. Subsequently, and surprisingly, it has been pointed out that even in normal conditions (in absence of iron overload) a non-vanishing part of free NTBI iron can be present. Than, the obvious queston follows: "how it is possible to find iron in its free form even when a formidable chelator as the transferrin is present?" A number of possible answers have been proposed to the above question. However, they appear to be partial, due to the numerous aspects not clearly defined and still object of investigation.

We begin focusing on the following point. The main sources of iron in humans derive from two fundamental mechanisms:

- a) the absorption by the intestinal mucosa (an exogenous food source);
- b) the recovery of iron past the lysis of aged erythrocytes and the macrophage activity (an endogenous source).

The average life of red cells is 120 days, During this time, they runs about 300, 400 Km and, once aged, they show ever more serious alterations, being eventually destroyed and their components recycled. In this way, the proteins are reused for different scopes, the iron is disassembled and reused by other cells to build red cells or any other molecules that require it. The released heme is degraded by the heme-oxygenase microsomal enzymatic system, that requires oxygen and NADPH and finally ends as bilirubin that enters the bowel and performs a function in the food digestion. The endogenous source of iron deriving from the previously described erythrolysis mechanisms is more relevant with respect to the exogenous one, as for the latter one the control and the absorption by the intestinal mucose is particularly selective and finely tuned.

As an example, in case of hemolitic anemia or of transfusion, the excess of erythrocyte lysis determines an increase of iron that binds its chelator, the transferrin, causing an increase of its saturation index and, at the same time, releasing a certain amount of free NTBI iron[18]. In different conditions, as for example in absence of transferrin, the circulating levels of NTBI iron can reach values of 20 µmol/L, while when the transferrin is present but in reduced quantity the previous values are, in general, less than 10 µmol/L[19]. It is well known that the iron is able to lead to an oxidative stress; on the other hand, the oxidative stress can release free NTBI iron from different sources like the "iron sulphur proteins", ferritin, hemoglobin, etc., as it has long been reported in the literature[20-23], and this observation should induce to reflection. In summary, the sources of free NTBI iron can be generated in different pathologic conditions in which to the availability of high level of iron but also when the levels of transferrin iron saturation are normal or low, as consequence of oxidative stress. In fact, during the oxidative stress, an amount of iron can also be released by stable sources (ferritin, heme), triggering a vicious circle[24,25].

In Table I we report some pathologic conditions in which circulating forms of free, unchelated iron are observed[26]:

In Table I it can be noticed that the higher free iron concentrations are not revealed in the hereditary hemochromatosis, as it could be expected, but in the cancer in course of chemotherapy[27]. Due to its cation nature, the iron easily binds to anion substrates giving rise to a number of new chemical compounds grouped, as already stated, under the denomination of non transferrin bound iron (NTBI). To the best of present knowledge, among all these substances in which the iron is readily available, the most frequent and dangerous are exactly those generated by the cation iron, like albumin, citrate, acetate, phosphate, etc. For example, in the hemochromatosis the more abundant form of NTBI if that one bound to citrate and acetate[28], the other form being that one bound to albumin (anion protein) also in conditions of transferrin poorly satured of iron[29]. These isoforms of NTBI iron[30] in which the Fe^{3+} is mainly bound to citrate and to albumines and, potentially, also to acetate, malate and phosphate, also increase the risk of infection[31] (indeed, it is known that the iron is used by bacteria, fungi etc. to improve their replication), as well as the toxicity due to the endocellular overload of NTBI that,

since has not the fine tuning mechanisms mediated by the transferrin, is able to directly enter the cell[32].

Pathology	Free iron form	Concentration ((µmol/l)
Hereditary hemochromatosis	NTBI	4.0 — 16.3
Thalassemia major	DCI	1.7 — 8.6
Diabetes mellitus	NTBI	0.62 ± 0.43
Severe renal impairment	NTBI	0.1 — 13.5
Cancer in course of chemotherapy	NPBI	10.6 ± 6.6

Table I - Pathologies and corresponding observed free iron forms (after Patel M, 2012, modified). NTBI - Non Transferrin Bound Iron, DCI - Desferrioxamine-chelatable iron, NPBI - Non Protein Bound Iron.

Modalities and problems related to NTBI iron determination

As previously discussed, it is clear that the NTBI iron forms that are not stably bound to carriers (transferrin, ferritin, heme, etc.) are numerous and probably not yet completely known. As a consequence, the methods used for their determination are correspondingly numerous and constantly updated. Basically, the techniques used are the following:

- 1- Inductive estimate or dosing, based on the use of glycopeptide antibiotics like the bleomycin used in oncology (10) (bleomycin detectable iron, BDI). In presence of iron, the bleomycin is able to degrade DNA, producing free radicals. By a complex method, introduced for the first time by Gutteridge et al.[33], it is possible to dose the iron not bound to transferrin.
- 2- Dosing by the use of different chelator agents, followed by the separation and dosing of the iron by analytic techniques (HPLC, atomic absorption, spectroscopy, etc.). During the years, different chelating agents have been used such as:
  - EDTA (ethylenediaminetetraacetic acid) Hershko and coworkers, 1978[34,35]
  - NTA (unsaturated transferrin)[36]
  - DCI: desferrioxamina chelable iron[11]
  - BPS: bathophenantroline-disulphonate, a chromogenic chelator able to detect free iron at 532 nm[12], etc.
- 3- Direct dosing of NTBI iron by fluorescence techniques, that are the most recent methods also with the help of siderophores like the azobactin[37] and those that are currently being used as routine [38,39].

The world's leading experts gathered on 2005 and on 2016 to compare the different techniques in view of their validation and standardization[40,41]. Despite a little preference for the fluorescence, an agreement has not yet reached towards the methods to be used as standard. In the last meeting[41] 60 samples extracted from patients affected by systemic iron overload (hemochromatosis, thalassemia, transfusion patients, etc.) have been sent to five labs all over the world and ten different laboratory kits have been tested for the determination of iron NTBI and LIP, without reaching, as we already said, an unique view, with an exception to be noticed, that is, the transferrin saturation index, that revealed to be the parameter in the best correlation to the possible presence of circulating NTBI-LIP iron. These researches are obviously performed on patients affected by systemic iron overload, that is, with transferrin highly saturated by iron, and not in pathologies with normal transferrin saturation, though in the literature[42] the presence of NTBI-LIP has been pointed out also in these circumstances. The iron that is not stably bound to its main carrier (the transferrin) represents a challenge to the different anti-oxidative mechanisms. Such NTBI iron enhances the oxidative stress mainly through the already cited Fenton and Haber-Weiss reactions (Figure 1): with an increase of the oxidative action via the different radicals that are produced (hydroxy, hydrogen peroxide, superoxide, hypoalose acids, etc.).

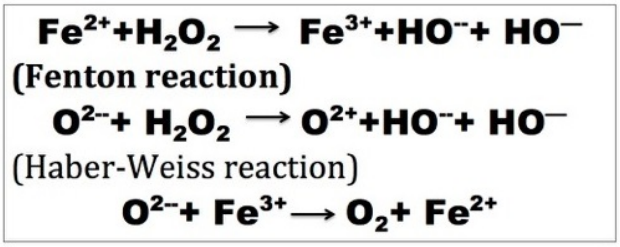

Figure 1 - Fenton and Haber-Weiss reactions.

Iron and free iron fraction in cardiovascular pathologies

One of the early remarks on the relation between iron and cardiovascular pathologies was made by Sullivan[43] on 1981. He pointed out how the lesser amount of iron deposits (ferritin, etc.) in menopause and post-menopausal women plays a protective role, as well as regular phlebotomy appears to do. Another randomized study shows, during an observation period of 5 years in a sample of 1931 subjects, the greater incidence (2.2 times) of myocardial infarction in patients with a ferritin level > 200 µg/l with respect to those

with a level <200 µg/l. A different work[45] points out, during a period of 6.4 years in a sample of 99 men, that subjects with low values of the ratio transferrin plasmatic receptor (*tfr*)/ferritin (that means large iron deposits) present a risk of myocardial infarction 2-3 times higher with respect to subjects with a larger tfr/ferritin ratio (Figure 2):

tfr/ferritin low → high iron deposits (iron storage)

tfr/ferritin high → low iron deposits (iron storage)

Figure 2 - Relation between transferrin plasmatic receptor/ ferritin ratio and iron storage.

Many works demonstrate the relation existing among iron and atherogenic progression.

- In the famous Brunico study[i], five-years long with a randomized sample of 125 men and 125 women age 40-79 years and a participation in the trial of 93.6%, a strong correlation between carotid asymptomatic atherosclerosis and iron deposits increase (particularly with a ferritin level >50 µg/L) is proven[46,47]. In[48] it is proved that in patients with end stage renal disease receiving an iron supply, is observed a thickening of the intima in the common carotid larger than in subjects that did not receive such a supply.
- A number of independent studies[49,50] show that in blood donors to low iron deposits levels correspond a lesser incidence of cardiovascular pathologies.
- The work[51] confirms a clear correlation in healthy individuals between the amount of iron deposits (iron storage) and asymptomatic carotid atherosclerosis studied by the ratio tfr/ferritin.
- In[52] (it is shown that chelation in patient affected by cardiovascular diseases is associated to an improvement of the endothelial function.
- The study[53] clarifies the relation between the iron food content and the coronary risk in a follow-up of 4 years and a cohort of 44933 men without any history of cardiovascular pathology, demonstrating the causal implication not so much with the iron quantity introduced with the food as with the heme iron content and the ferritin. Recently, in a meta-analysis of 21 valid studies Hunnicutt[54] remarks the correlation among dietary iron, iron storage and coronary risk. The dietary iron considered in the finnish work by Salonen[55] appears to be correlated in a significant way to the coronary disease. The author reports that an increase of 1.0 mg of dietary iron corresponds to an increase of 5% of the coronary risk. The heme iron contained in the meat (myoglobin and hemoglobin iron) represents about the 40% of the food iron and, thanks to its high bioavailability[56] its absorption is not inhibited with the negative feedback by the high levels of circulating ferritin, as happens to non-heme iron (inorganic iron)[57]. Non-heme iron can also be found in meat, lever, egg yolk, vegetables, etc. and is present both as Fe^{2+} and Fe^{3+} form. Recently, Wolk[58] proved by meta-analysis a significant increase of cardiovascular mortality after daily intake of 50 g meat, as already observed by Micha[59] in relation with treated meat compared with fresh ones.
- Another point worth of attention is that all agents which inhibit the dietary absorption of iron (polyphenols, soy, casein, whey, chicken egg albumen, etc.) affect non-HEME iron only. Furthermore, adding Calcium does not affect the absorption of neither HEME nor non-HEME[60] iron, in spite of what has been reported during previous studies (Figure 3).
- According to some authors[61] exercising can in many ways play a major role in iron excretion.
- Some studies show that there is a correlation between cardiovascular conditions and iron[62-65].
- Very interesting experimental studies[66-68] show that adding NTBI iron to cultures of human endothelial cells increases the expression of VCAM 1 (Vascular Cell Adhesion Molecule, etc.) as well as leucocyte rolling and endothelial barrier damage. This phenomenon can be stopped by adding iron chelating agents (desferrioxiamine, dipirydyl) to the cultures of human endothelial cells[67], as it is also confirmed by another study on endothelial cells in the human aorta[68].

There are more studies confirming the correlation between iron and cardiovascular condition compared to those which deny it[68-71], and albeit scientists are still divided concerning this topic, it is necessary to highlight that many of the studies which refused this correlation have been carried out with inappropriate techniques and indicators.

More recent studies[72] verified that NTBI can play a major role in terms of cardio toxicity in myocardial infractions (STEMI: ST evaluation acute myocardial infraction), especially as far as MVO (Micro Vascular Obstructions) and HEM (Hemorrhage) are concerned, the latter being a source of NTBI.

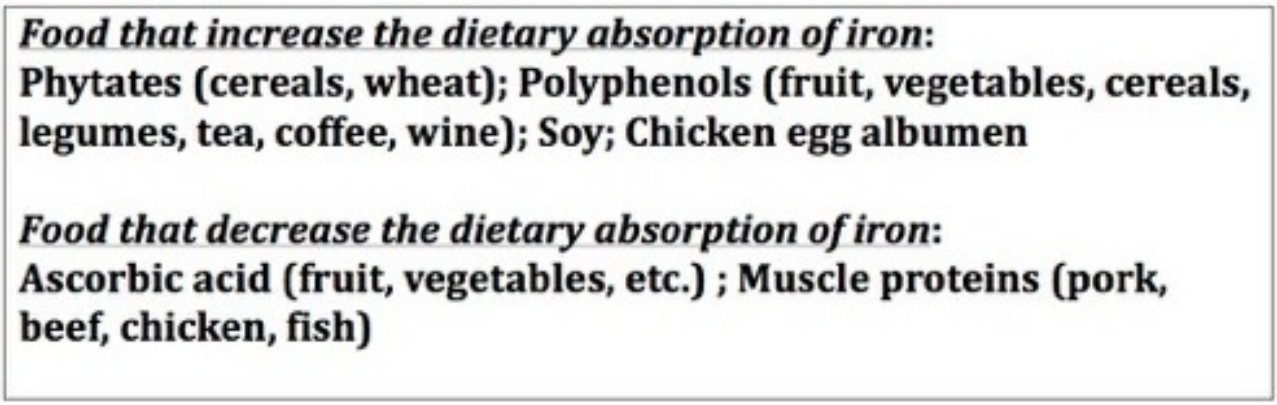

Figure 3 - Food causing increase or decrease of dietary iron absorption.

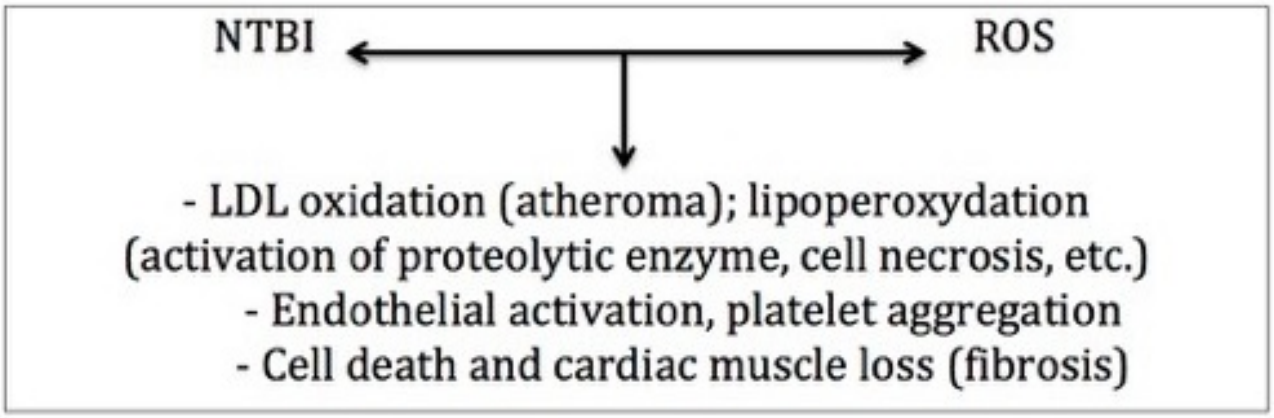

Figure 4 - Cardiovascular toxicity of NTBI iron.

This study points out the close ties between NTBI, CK-MB and troponin; even though the number of patients was rather scarce, such a correlation will definitely be the object of more and more thorough studies.

The study FeAST highlights that even chronic obstructive arterial disease in lower limbs is correlated with iron[73]; furthermore other randomized experiments point out that the mere phlebotomy significantly reduced the mortality rate (myocardial infraction, stroke) among patients suffering from chronic obstructive arterial disease in lower limbs[74].

The cardiovascular toxicity of NTBI can be summed up as in Figure 4.

In one of his most recent papers[75], Brissot highlights that even though there is not yet full knowledge of every factor able to produce NTBI, a major role is played by the iron loss in the transferrin-ferritin, by a poor iron re-usage during the erythrocyte lysis and, even more alarmingly, by the condition of chronic oxidative stress.

Other authors[76,77] highlight that NTBI is a valid marker as far as biological damage is concerned, and not only in iron overload syndromes.

The main factor which regulates the iron output of a cell is the ferroportin under the effect of plasma hepcidin; therefore, low levels of hepcidin can trigger high level of transferrin saturation and NTBI iron, which is easily absorbed by the cells.

Recently Riško[77] shows that patients with chronic cardiovascular conditions (myocardial infraction, stroke, peripheral obstructive arterial disease) present monocytes free iron-LIP concentration higher than usual; such an anomaly is statistically correlated to the following parameters:

- TfR/F (ratio: circulating transferrin receptor/ ferritin)
- Hepcidin plasma values
- Visceral fat (insulin-resistance)
- ABI index (Ankle-Brachial Index or Winsor index, i.e. an index used in obstructive arterial disease in lower limbs)

- Arterial stiffness (Pulsatility Index (PI) and Resistance Index (RI))

Such parameters, therefore, highlight the possibility that the intra-monocyte LIP (Labile Iron Pool) can be a sensitive index of the risk of atheroma formation.

Nevertheless, it is important to remark that though the lack of iron is related to cardiovascular disease, the study ARIC[78] on anemic patients show that "*virtue stands in the middle*". The ratio of body iron concentration and risk of occurrence of cardiovascular condition is not linear; on the contrary, it can be described as a U-shaped curve[79].

Iron, iron-free and atherosclerosis

Over the past 30-40 years many studies[5,24,42,46,48,51,62,66,67,77,78] highlighted how the iron mediated oxidative stress played a major role in the formation of the atherosclerosis, also recently mentioning the toxicity of hemoglobin-free (Hb-free) and its iron content derived from erythrocyte lysis[79].

For years the iron-induced oxidative stress was considered to be the main pathogenic trigger of atherosclerosis. Today, however, it is known that the factors that trigger the formation of atheroma are many and complex[80], as far as iron and NTBI are concerned.

Specifically, hydroxyl radicals oxidize lipids and proteins, causing endothelial dysfunctions, cell proliferation (activation of monocytes- macrophages), DNA and immune system damage.

The oxidation of the low density lipoprotein (LDL) is a focal point in the formation of atheroma.

The reactive oxygen species (ROS) can alter the triglyceride fatty acid chains, especially the unsaturated ones, cholesterol esters, and protein structures with formation of Malondialdehyde (MDA) peroxides or hydroxides, pentanes, etc., the oxidation output of the cholesterol, i.e. oxysterols deriving from phospholipids, the alteration of apolipoprotein into carbonyls or amino acids (cysteine, cysteine, histidine, etc.), the formation of lipids-protein structures known as lipofuscin[81].

Chemical analysis of lipofuscin samples shows the presence of protein (20-50%) and lipid (30-70%) components. The protein component can vary, whereas the lipid component is composed by triglycerides, saturated fatty acids, cholesterol, phospholipids carbohydrates and metals, especially iron[82].

The LDL oxidation represents a crucial moment in the process of the formation of atheroma, triggering:

- 1 endothelial activation and dysfunction;
- 2 macrophages activation and transformation into foam cells;
- 3 adaptive modification in the immune response.

Most of the studies on the LDL oxidation (Ox-LDL) reported in vitro experiments where pro-oxidizing agents, such as iron, were used. So far scientists are not certain that the LDL oxidation occurs mainly on arterial walls, even though this is almost always the case for patients suffering from diabetes or cardiovascular diseases[83]. Moreover, the LDL oxidation appears to be correlated to the rise of the C-reactive protein (CRP)[84].

It appears that the endothelial dysfunction triggers the cell adhesion molecule (I-CAM 1 or E-Selectine); as a result, the circulation monocytes are then divided in two groups, one composed by the monocytes trapped in the endothelium and the other one by the monocytes which flew through the sub-endothelial space. This grouping takes place under the effect of cytokines such as M-CSF (Macrophage Colony Stimulating Factors, produced by macrophages themselves), endothelium cells and T lymphocytes, macrophages, specifically macrophages M1 (pro-inflammatory) rather than M2 (anti-inflammatory)[84].

Macrophages M1 express Ox-LDL receptors on their surface for Ox-LDL that, once absorbed, turn the macrophages into foam cells due to the accumulation of cholesterol esters, etc. This macrophage population would also be the cause of both the proliferation of smooth muscle cells that migrate towards the arterial endothelium, and of the atheroma instability due to the activation of metalloproteinase 1,3,9 (MMP 1-3-9) with hydrolysis of the collagen in the atheroma fibrous cap[85,86].

M2 macrophages have opposite tasks compared to the M1, and are also able to trigger several metalloproteinase (MMP 9-12-13-14) that are important in the re-shaping of the atheroma. Compared to M1 macrophages, M2 macrophages have a lower iron concentration [87]. In addition, chelating substances, such as the lactoferrin, can play a protective role[88]; furthermore, the M1 activation can be directly induced by iron[89].

Another factor that has recently been studied is the red blood cells phagocytosis in hemorrhage areas, specifically in atheroma[90]. This phenomenon is an important factor determining iron accumulation in Mhem macrophages that have features similar to the M2 macrophages (protective)[91,92]. These HEME filled macrophages also present high HMOX1 levels (heme oxygenase-1, an enzymatic form which, contrarily to HMOX2, is inducible) that can trigger M1 macrophages (pro-inflammatory).

The heme oxygenase is an oxidoreductase that catalyzes the transformation of the heme into biliverdin, carbon monoxide and iron. Thanks to the biliverdin

reductase, the biliverdin is later on transformed into bilirubin. The iron released induces the expression of the intracellular apoferritin with iron sequestration from the ferritin and iron redox reduction, as a result of the protective effect of the ferritin.

The heme oxygenase appears to play a crucial role in the cellular iron metabolism regulation; furthermore, it is an enzyme that can be induced by many factors such as oxidative stress, inflammation, reperfusion, etc. It is worth being noted, however, that free heme can directly activate M1 macrophages by interacting with Toll-like receptors expressed by many cells (monocytes, macrophages, mast cells, etc.), TLR4 (Toll-like Receptor 4) and innate immunity cells. Contrarily to HMOX2, the enzyme HMOX1, (heme oxygenase 2) is an inducible stress enzyme and its oxidoreductase regulates vascular activity and prevents inflammation and formation of atheroma[93].

As a matter of fact, HMOX1 polymorphism or deficiencies can be linked to the occurrence of many diseases, especially atherosclerosis, cardiovascular disease, etc.[94,95].

Another study[96] shows that the iron contained in the atheroma is a risk factor which can be modified as far as the formation of atheroma plaques is concerned.

Iron and free iron in the chronic venous insufficiency

In the advanced stages of chronic venous disease that can be considered as insufficiency (CEAP Classification: C3-6)[97,98] after the occurrence of edema (venous edema, CEAP: C3) a progressive tissue dystrophy takes place, usually evolving to ulcers. This kind of dystrophy is named lipodermatosclerosis (LDS, CEAP: C4b) and it is characterized by the progressive accumulation of hemosiderin iron in dermis and hypodermis. Legs undergo a hyper pigment production and hardening transformation, known as "inverted champagne bottle leg". Lipodermatosclerosis was initially described in 1955 by Huriez as *Hypodermitis sclerodermiformis*[99], and later on many other authors denoted this condition by different names (liposclerosis, sclerosing panniculitis, lipomembranous panniculitis associated to venous stasis, etc.). However, this condition is today known in the scientific literature with the name of Lipodermatosclerosis (LDS).

The inferior limbs' chronic venous stasis triggers ambulatory venous hypertension and, finally, ends up rising the transmural pressure, with the appearance of erythrocyte extravasation (extravascular hemolysis)[100-102] which is also cause of the local hemosiderosis[103] due to hemosiderosis-iron overload.

More recent studies[104-107] have progressively showed and clarified that the hemosiderosis-iron overload levels reach their peak in the chronic phase of the lipodermatosclerosis, especially subcutaneously, and then fall due to a progressive fibrotic substitution. Other studies where MRI was used[108] demonstrated the progressive reduction of subcutaneous fat and its peculiar "honeycomb pattern". The necrosis appears to affect both the adipose lobe and the inter-adipocitary septum (lobe-septum necrosis); the iron can directly trigger lipolysis due to the oxidative stress[109].

The hyperpigmentation occurring in lipodermatosclerosis has some distinctive points:

- melanine accumulates both in the *stratum basale* and in the dermis; melanocytes migrate towards the dermis[110];
- hemosiderin sediments in dermis and hypoderm[107,111] (107, 111);
- melanocyte melanin synthesis appears to be secondary to iron overload and triggered by oxidative stress and iron mediated oxidative stress[112-114];
- melanin acts like a powerful iron chelating agent[115,116] and the melanocyte migration towards the dermis might be due to the melanin chelating activity (biological defense strategy).

The correlation between the iron overload in tissues and venous ulcers in the lower limbs was reported for the first time by Ackerman in 1988[117], which observed that the consistent iron hyper overload in the ulcerated tissues was about 10 to 15 times higher compared to the same area in the counter lateral limb.

The presence of hemosiderinuria[118] is later reported in advanced dystrophic-ulcerative stages of chronic venous insufficiencies and, later on, in other disorders such as lymphedema, connective tissue disease, but not in ischemic ulcers[119].

Other authors[120] highlight that iron is present in the ulcer fluid taken from chronic ulcers in lower limbs, but not in that extracted from acute ulcers.

Further studies highlight the relation between tissue iron overload and venous ulcers and some HFE polymorphism (hemochromatosis gene)[121-125] and also provided therapy evidence by using topical chelating agents which fasten the venous ulcer healing process[126,127]. Some authors[128] used the PIXE (Proton Induced X-ray Emission Spectroscopy) to highlight that the iron concentration rises with the evolution of the disease; in fact, the iron deposition rate is higher in incompetent veins compared to competent veins (surgically collected veins); this rate is statistically

correlated to oxidative stress parameters and to the ratio *intima-media*. The conclusions of these studies are that the dosage of the PIXE iron can be correlated to the evolution of the insufficiency.

Ferritin and hemosiderin represent the main ways iron can sediment; they differ each other as follows:

- hemosiderin is not water-soluble, but undergoes denaturation;
- ferritin is water-soluble but is temperature resistant up to 75°C (167°F).

Hemosiderin iron is stored in the internal part of the protein shell, especially in the form of ferric hydroxide ($Fe(OH)_3$) as a consequence of its degradation and lysosome polymerization[129]. Ferritin is normally the predominant way in which iron is stored in the tissues; hemosiderin, however, can become the predominant form in case of iron overload, causing biological damage[130] (Figure 5).

In fact the hemosiderin, especially in acid conditions (inflammation, ischemia), can release iron enhancing oxidative stress. When these conditions occur, the hemosiderin can turn itself from iron chelating agent to an iron releasing agent in the form of sediment:

For example, iron can sediment due to erythrocyte extravasation (erytrhodiapedesis, hemolysis related causes, etc.) both in ferritin and hemosiderin (Figure 6). Moreover, iron can be directly be mobilized by hemosiderin or ferritin[130-134].

An interesting study on animals[135] was led by inducing skin iron overload in three different ways (HFE mutation i.e. hemochromatosis, dietary iron overload, direct iron injection); the results of the study highlight that the direct iron injection is the most harmful of the three.

Another study[136] highlights the direct correlation between the stage of skin exfoliation (dyskeratosis) and the level of iron in the skin. Yet another study analyses the toxic effects derived from iron overload in many diseases, including connective tissue disease (Lupus, rheumatoid arthritis)[137] and in the wound healing process.

Finally, a biopsy study[138] was led on many cases of lower limb dermatitis (lichenoid, tinea, chronic venous stasis, folliculitis, psoriasis, etc.) where the Gomori test was used to determine the iron levels in the tissues; the results of the test were positive in 42% of the cases, thus highlighting the role iron plays in many chronic inflammatory skin disease.

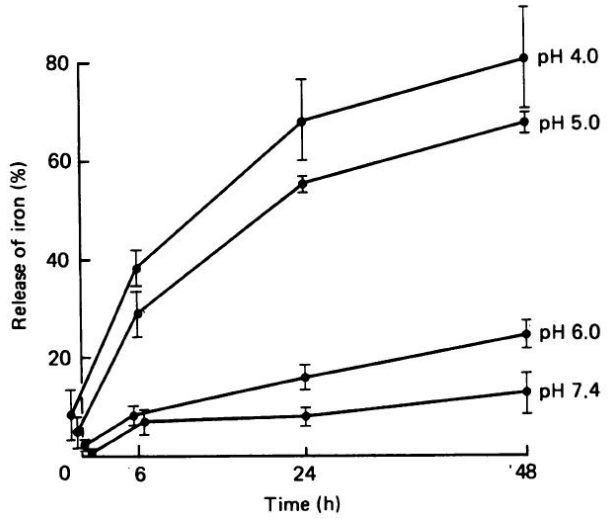

Figure 5 - Iron release vs time (Ozaki 1988, with permission).

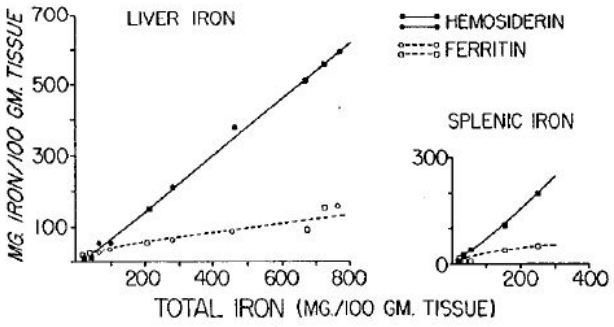

Figure 6 - Iron forms vs total tissue iron content (Shoden 1953, © the American Society for Biochemistry and Molecular Biology).

Conclusions

Many authors studied the whole literature concerning the relation between iron accumulation and the increased occurrence of cardiovascular diseases[139]; even though this topic is still under discussion, the new techniques in the dosage of free NTBI highlighted the role played by free hemoglobin and HMOX1 (heme oxygenase) in the regulation of the cellular iron and the inducing effect of some drugs on the HMOX1, such as statins, aspirin and more particularly lansoprazole, etc.[140,141], their protective effect in the vessel endothelium[142] and, lastly, the challenge

of the chelating therapy in local siderosis[143]; all of the abovementioned factors seem to corroborate the hypothesis that the iron overload is toxic in cardiovascular diseases.

Chronic venous insufficiency is an ideal case to be investigated by studying NTBI as the hemosiderin deposition is very consistent.

Endnotes

[i] Brunico (Bruneck) in the province of Bolzano (Bozen), Italy, at the border between Italy and Austria.

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VENOUS HEMODYNAMICS ORIGINAL ARTICLE

Experimental validation of the Paraná manoeuvre compared to the squeezing test

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Abstract

Background and aim

The squeezing test (ST) is widely practiced, owing to its simple execution. In 1997, the Paraná (P) manoeuvre was proposed. This manoeuvre consists in a gently pushing-from-the-rear or pulling-from-the-front. Our aim was to compare the hemodynamic effects of ST and P during the muscle systole and diastole.

Method

57 patients underwent a diagnostic Duplex examination. 57 legs were examined, one leg for each patient, exploring just one venous segment for each leg. 37 patients were affected by incompetence of the terminal valve of the saphenous-femoral junction. 20 patients presented just telangiectasiae (C1) and were used to compare the manoeuvres in competent popliteal veins. Measurements were taken on 57 venous segments (20 competent popliteal veins, 13 incompetent saphenous-femoral junctions, 13 incompetent trunks of great saphenous vein and 11 re-entry perforating veins). Comparisons were worked out using a two-tailed paired t-test.

Results

Compared to ST, P moves 68% more blood volume in systole in the competent popliteal vein ($p=0.00014^{***}$), while the diastolic phase of P is 2.52 times longer in incompetent SFJ ($p=0.00003^{***}$), 1.83 times longer in the incompetent GSV trunk ($p=0.0015^{**}$) and 3.27 times longer in the re-entry perforating veins ($p=0.07$ n.s.). However, this last result, near to significance, needs further investigations. In addition, our data about the systolic acceleration did not show any meaningful result.

Conclusion

P is a better test than ST in the evaluation and quantification of reflux and could be of paramount clinical importance in improving diagnostics in venous diseases, being actually practised since 20 y in many vascular labs. P does not rely on the size of the operator's hand or the size of the patient's calf and investigates a condition which is almost near to the physiological posture balance.

Keywords Paraná, squeezing test, venous hemodynamics, dynamic manoeuvres.

Background and aim

The ultrasound assessment of the venous reflux has an important role in the examination of the venous function of the lower limbs. The conventional calf compression and release manoeuvre or squeezing test (ST) is generally practiced in many vascular labs and is performed by a sequence of compression and release of the calf, with several variants constituted for instance by the

manual compression or instead the standardized pneumatic compression by means of a cuff[1-4]. Its execution is generally simple, one of the limitation being given by a big calf circumference, which cannot be easily compressed by a small hand. However, ST does not correspond to any daily activity and its results cannot be interpreted as an answer to any physiological solicitation.

In 1997, the Paraná (P) manoeuvre[5,6] (Figure 1 A & B) was proposed, which is constituted by a gentle push-or-pull manoeuvre, taking its name from the city of Paraná (Argentina), where it was conceived. Gently pushing-from-the-rear (Figure 1A) or pulling-from-the-front (Figure 1B) at the patient#s waist, the manoeuvre elicits a proprioceptive reflex in order to maintain the balance. An almost isometric contraction (systole), mainly of the calf but also of the thigh, pelvis and lumbar spine, is then followed by a relaxation (diastole). The contraction is not purely isometric, because consequently a re-equilibrating movement is generated. In systole a centripetal flow in the venous system occurs, followed by a zero flow or a reflux when valves are respectively competent or incompetent.

The aim of this paper is to compare the hemodynamic effects on flow of ST and P during systole and diastole in the examined venous segments[7].

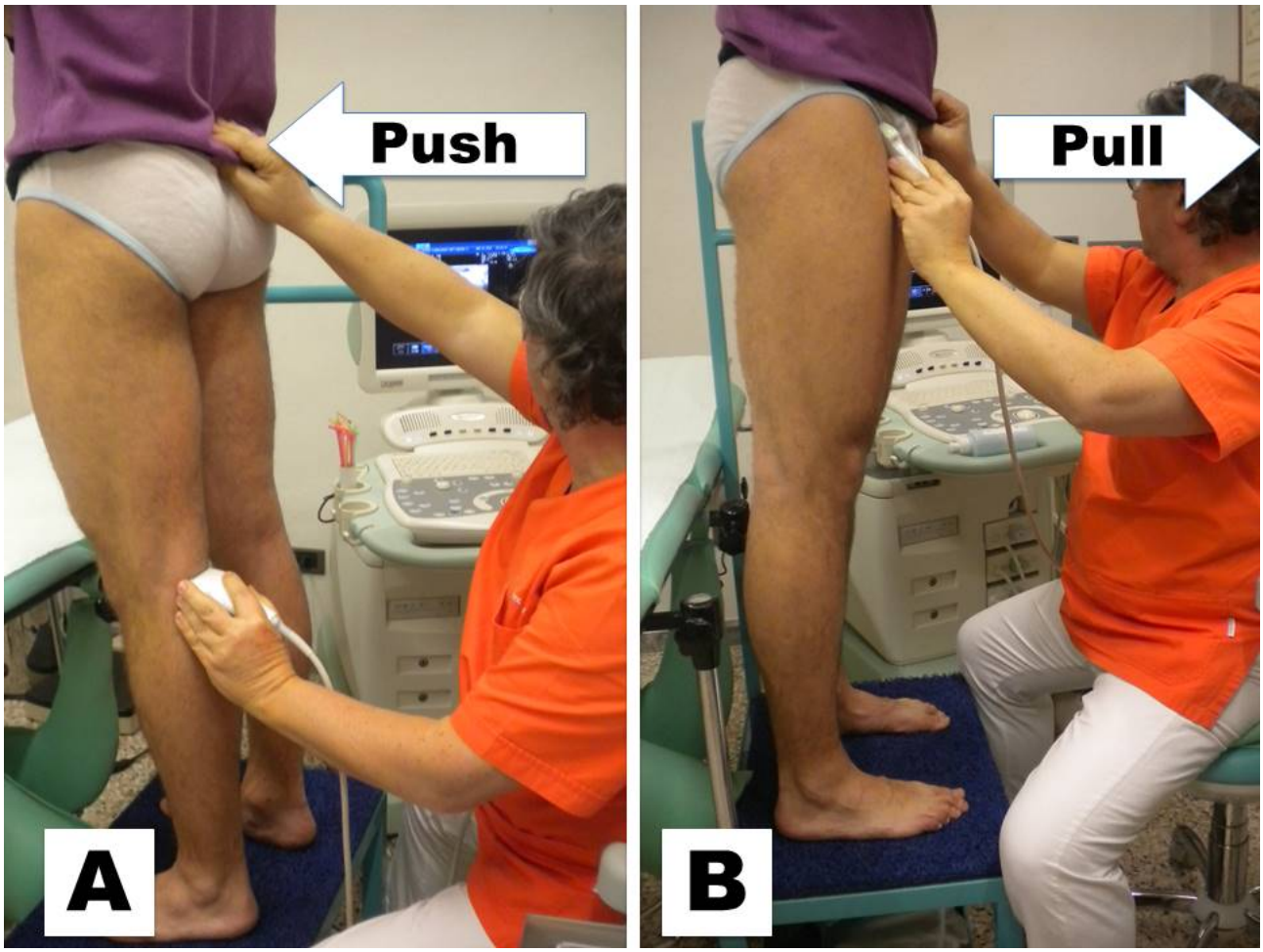

Figure 1 - The patient and the operator's positions for the 'push-pull' during the Paraná manoeuvre: push from rear at the popliteal level (A) and pull from front at the GSV trunk and perforating veins level (B).

Patients and methods

Device and settings

An Esaote Mylab 50 ultrasound device equipped with a 12 Mhz linear probe was adopted. The probe was positioned longitudinally and the best image was captured. The steering angle was then adjusted in the range 30°-45° and the sample volume width and the Doppler angle were adapted to the scanned vein.

	Measurements		
	# cases (1 leg / patient)	# measurements/leg	# measurements
Competent popliteal veins	20	6 (vmaxS, T_{S1}, T_{S2} for each manoeuvre)	120
Incompetent SFJ	13		104
Incompetent GSV trunks	13	8 (vmaxS, TS, vmaxD, TD for each manoeuvre)	104
Re-entry perforating veins	11		88
Total	57		416

Table I - Legs / venous examined segments and performed measurements.
SFJ saphenous femoral junction, GSV greater saphenous vein, $vmax_S$ max systolic velocity, T_S systolic ejection time, T_{S1} systolic acceleration time, T_{S2} systolic deceleration time, $vmax_D$ max diastolic velocity, T_D diastolic refluxing time.

Patients

A consecutive sequence of 57 patients underwent a diagnostic Duplex (DUS) examination. All patients signed a written consent to allow non-invasive ultrasound measurements. 57 legs were examined, one leg for each patient, exploring just one venous segment for each leg. 37 patients were affected by incompetence of the great saphenous vein (GSV) with insufficiency of the terminal valve of the saphenous-femoral junction (SFJ). There were no healthy volunteers in this study, but 20 patients presented just telangiectasia (C1) with deep and superficial veins competence and were used to compare the ST and P manoeuvres in competent popliteal veins.

Manoeuvres

The ST manoeuvre was performed manually, according to the modality generally practiced in almost all vascular labs.

The P manoeuvre was effected from rear when measuring on PV, from front when on SFJ or GSV and in a variable way when on a re-entry perforating vein, according to its anatomical site. For instance, from rear when measuring on a perforating vein of the calf, from front when measuring on a medial perforating vein along the saphenous channel.

Each manoeuvre was divided into phases: the evocation phase or systole, the rest phase or diastole:

- ST: compression was the systole, relaxation the diastole.
- P: push/pull was the systole, the spontaneous postural adjustment the diastole;

Measurements and computations

Measurements were taken on 20 competent popliteal veins (PV), 13 incompetent SFJs, 13 incompetent GSV trunks and 11 re-entry perforating veins. All observations were performed by the same operator, thus this study was designed in a simplified form, not gathering at all intra-observer differences.

In order to avoid mutual influences among data in the same patient, measurements were taken in standing position just on one lower limb and just on one venous segment per patient, with a total of 57 segments: PV measurements in the clinical classes C0-C1, non-PV measurements in the clinical classes C2,C3,C4, i.e. no trophic lesions, neither open nor closed, in the observed sequence.

On the total of 114 segment records (57 × 2, i.e. for both P and ST) 416 measurements were performed (Table I).

In all the examined veins, the requirement for the valve incompetence was a time length greater than the threshold value of 0.5s

In the SFJ the valve incompetence was assessed adjusting the Doppler direction perpendicular to the terminal valve plane, (Figure 2) whilst for the GSV trunk the sample volume was set 15 cm below the groin and the Doppler direction adjusted along the saphenous axis. All measurements were performed adopting a longitudinal section of the investigated venous segment.

A re-entry perforating vein was defined as a connection between the superficial and the deep system, fed by the GSV reflux and entering into the deep veins of the leg, having an anterograde superficial-to-deep (-) diastolic velocity, while the systolic velocity could be anterograde i.e. re-entering (-), null (0) or refluxing (+).

In a re-entry perforating vein, the blood re-entering in diastole is generally much more of the blood eventually directed outward in systole. Measurements were taken at the most linear part of the perforating vein.

The time length (s) of the velocity curve in systole (TS) and diastole (TD) was measured. In addition, TS was subdivided into TS1, the acceleration time, and TS2, the deceleration time (Figure 3).

The systolic acceleration (a_S) ($cm \cdot s^{-2}$) was computed dividing the max systolic velocity Vmax by T_{S1}

$$\mathbf{a_S = Vmax/T_{S1}}$$

The area under the velocity curve (AUC) (cm) was computed according to the formula:

$$\mathbf{AUC_S = T_S \cdot Vmax_S /2}$$

$$\mathbf{AUC_D = T_D \cdot Vmax_D /2}$$

$$\mathbf{AUC_{S+D} = AUC_S + AUC_D}$$

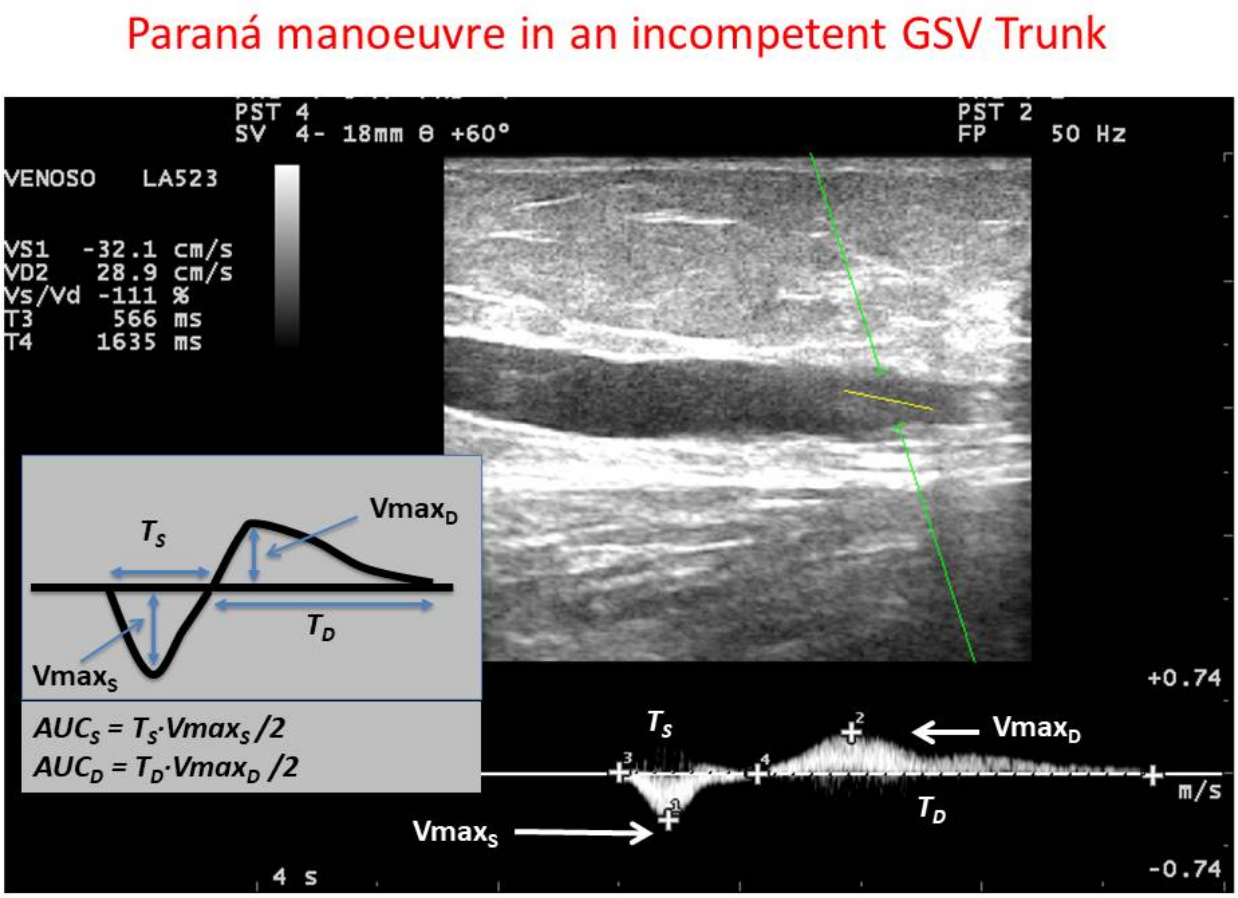

Figure 2 - The Paraná manoeuvre in an incompetent GSV trunk. The measurements were taken 15 cm below the groin. GSV greater saphenous vein, T_S systolic ejection time, T_D diastolic refluxing time, Vmax max systolic velocity, AUC area under the curve.

The above formulas could be theoretically justified using two equivalent methods: estimating the mean velocity Vmean = Vmax /2 (cm·s-1) or, in an equivalent way, computing the area of the triangle with base=time and height=Vmax.

Although the AUC is graphically an area, it assumes a special meaning instead, according to the adopted graphical representation. Taking into account the dimensions of the variables which are reported in the current graph, the AUC has the dimension of a length (cm). Assuming a constant venous calibre in the same subject and in the same position, the non-dimensional AUC ratio was considered numerically equivalent to the non-dimensional volume ratio, the calibre being cancelled in the math operations.

Indeed, the AUC (cm) was considered dependent in a linear way and through the (assumed constant) area section on the mobilized blood volume (cm^3). Thus, in the comparisons ST *vs.* P, the AUC values could be interpreted as blood volume values, ejected in systole or refluxing in diastole, i.e. making it possible to compare the efficacy of the two dynamic manoeuvres. The essential point is that the measurement of the cross sectional area is not needed for the comparison, as the assumed constant area is cancelled in the ratio computation.

Statistics

Each measurement was expressed as a value ± sigma (the standard deviation). Each mean was expressed instead as a value mean ± se (the standard error of the mean). The coefficient of variation cv was given by the ratio cv sigma/mean. These notation choices did not influence at all the statistical computations, which were applied using the required computations and all the available data.

The notations $p<0.05$(*), $p<0.01$(**), $p<0.001$(***) and n.s.(not significant) were adopted, though only the first threshold was used for statistical significance.

The ST and P manoeuvres were performed in sequence and the order of application was chosen randomly, with an adequate resting period, approximately worth twice the diastolic refluxing time. Data from ST and P were compared in order to underline any difference in the hemodynamic effect of each manoeuvre.

The comparison between ST and P results was effected by a two-tailed paired t-test, while a two-tailed F-test caught the inequality of variances in the two groups. NB! the F test has an asymmetrical distribution, thus F tests are generally one-tailed, dealing with the variance ratio of ordered groups of data. A two-tailed F test is a one-tailed F test, performed when no hypothesis is formulated in advance about which of the two groups has the greater variance, i.e. the greater variance is always divided by the smaller one or more simply groups of data are non-ordered.

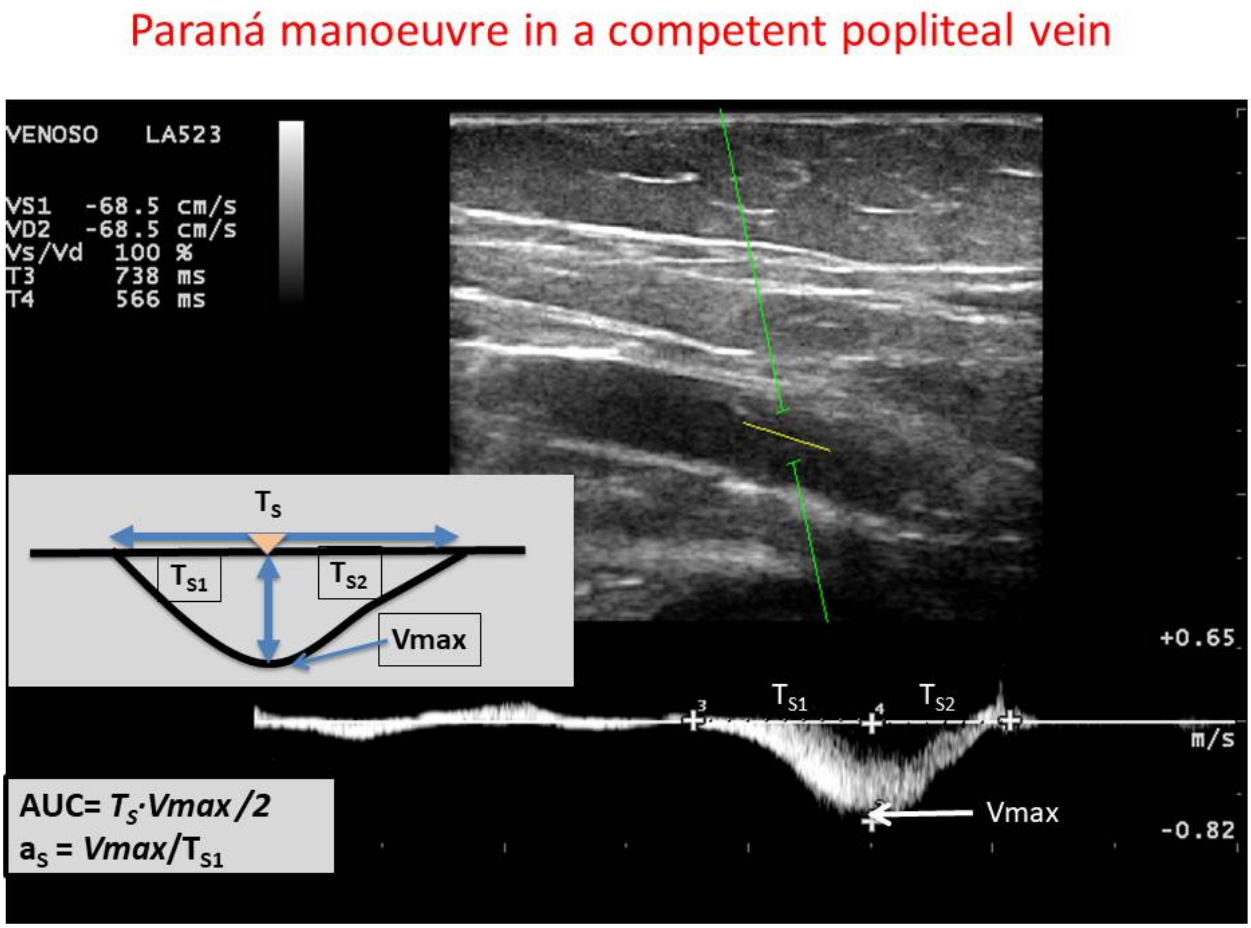

Figure 3 - The Paraná manoeuvre in a competent popliteal vein. The sample volume is adapted to the venous size and the steering line set in the range 30° - 45°. The Doppler angle is adapted according to the vessel direction. T_S systolic ejection time, T_{S1} acceleration time, T_{S2} deceleration time, Vmax max systolic velocity, AUC area under the curve, a_S systolic acceleration.

Competent popliteal veins		
	ST	P
Systolic T_{S1} (ms)	380±20	590±40
Systolic T_{S2} (ms)	350±14	550±30
Max Systolic Speed ($cm \cdot s^{-1}$)	68±5	72±7
Systolic AUC (cm)	25±2	42±4

AUC = $(T_{S1} + T_{S2}) \cdot$Max Systolic Speed/2000 - Systolic AUC P/ST = 1.68, p=0.00014**

Table II - Systolic blood volume ejected in 20 competent popliteal veins.
T_{S1} systolic acceleration time, T_{S2} systolic deceleration time, AUC area under the curve, P Paraná, ST Squeezing Test. Mean ± standard error of the mean.

Results

Systolic Popliteal flow

The systolic acceleration measured in the competent popliteal veins (Figure 3) showed a smaller average value of 0.13 $cm \cdot s^{-2}$ for P and 0.19 $cm \cdot s^{-2}$ for ST ($p=0.014^{**}$) but P moved 68% more blood volume ($p=0.00014^{***}$) (Table II). In addition, the popliteal AUC in systole was systematically greater than the AUC in any other superficial vein. The comparison was possible only in systole and not in diastole, as all investigated popliteal veins were competent.

Time length of the diastolic phase

In incompetent superficial veins, the diastolic phase of P, compared to ST, was 2.52 times longer in the incompetent SFJ ($p=0.00003^{***}$) (Table III), 1.83 times longer in the incompetent GSV trunk ($p=0.0015^{**}$) (Table IV) and 3.27 times longer in the re-entry perforating veins (p=0.07 n.s.) (Table V), though this ratio was not enough to reach the significance.

Incompetent SFJ - systolic and diastolic flows

Terminal valves were incompetent in all examined SFJ. P produced a slightly greater systolic ejection volume 1.21 times than ST (p=0.19 n.s.). In addition, P gave a consecutive diastolic reflux volume 3.77 times greater ($p=0.009^{**}$) (Table III).

Incompetent GSV- systolic and diastolic flows

In the incompetent GSV trunk (Figure 3), P produced a slightly greater systolic ejection volume 1.42 times than ST (p=0.59 n.s.). In addition, P gave a consecutive diastolic reflux volume 2,0 times greater ($p=0.02^{*}$). (Table IV).

Re-entry perforator - systolic and diastolic flows

In the re-entry perforating veins, P produced a slightly greater systolic outward reflux volume 1.62 times than ST (p=0.08 n.s.), in addition providing a diastolic inward volume 4.84 times greater ($p=0.009^{**}$) (Table V).

Systolic acceleration

The systolic acceleration was slightly smaller for P vs. ST in the competent popliteal vein ($p=0.014^{*}$) and in the incompetent SFJ ($p=0.008^{**}$) (Table VI), while in the other veins differences were negligible. The standard deviation of the systolic acceleration for ST was greater in the incompetent SFJ ($p=0.0002^{***}$) and smaller in the incompetent GSV ($p=0.0000001^{***}$), while in the other veins differences were negligible. Our data regarding the systolic acceleration were somewhat contradictory, suggesting that no unique interpretation about accuracy could be formulated in this patients' series.

Discussion

It should be noted that we performed all measurements in longitudinal section more fit to quantitative research. In daily clinics instead the Paraná manoeuvre is generally used with both longitudinal and transverse oblique sections (Figure 1).

Intra-observer differences were not investigated. Our data demonstrated that P caused greater flow variations than a conventional ST. Greater volume shifts were observed and therefore it could be inferred that P is a better test than ST in the evaluation and quantification of reflux.

Incompetent SFJs

		ST	P
Systolic events			
	Systolic T_S (ms)	750±60	1140±80
	Max Systolic Speed S ($cm \cdot s^{-1}$)	45±6	34±4
	Systolic AUC (cm)	16±2	19±3
Diastolic events			
	Diastolic T_D (ms)	2300±200	5800±600
	Max Diastolic Speed D ($cm \cdot s^{-1}$)	43±9	70±20
	Diastolic AUC (cm)	49±13	185±40

Systolic AUC P/ST = 1.21, p=0.19(n.s.), Diastolic AUC P/ST = 3.77, $p=0.009^{**}$ Diastolic TD P/ ST = 2.52, $p=0.00003^{***}$

Table III - Analysis of the Paraná Manoeuvre and Squeezing Test in 13 incompetent SFJ.
SFJ saphenous femoral junction, T_S systolic ejection time, T_D diastolic refluxing time, AUC area under the curve, P Paraná, ST Squeezing Test. Mean ± standard error of the mean.

Incompetent GSV trunks			
		ST	P
Systolic events			
	Systolic T_S (ms)	500±40	570±140
	Max Systolic Speed S ($cm \cdot s^{-1}$)	30±6	26±5
	Systolic AUC (cm)	7,7±1,1	10±4
Diastolic events			
	Diastolic T_D (ms)	4300±700	7900±1100
	Max Diastolic Speed D ($cm \cdot s^{-1}$)	35±5	41±9
	Diastolic AUC (cm)	70±10	140±30

Systolic AUC P/ST = 1.42, p=0.59(n.s.), Diastolic AUC P/ST = 2.0, p=0.02* Diastolic TD P/ST = 1.83, p=0.0015**

Table IV - Analysis of the Paraná Manoeuvre and Squeezing Test in 13 incompetent GSV trunks.
GSV greater saphenous vein, T_S systolic ejection time, T_D diastolic refluxing time, AUC area under the curve, P Paraná, ST Squeezing Test. Mean ± standard error of the mean.

Re-Entry Perforating veins			
		ST	P
Systolic events			
	Systolic T_S (ms)	470±40	760±120
	Max Systolic Speed S ($cm \cdot s^{-1}$)	28±5	26±3
	Systolic AUC (cm)	6,6±1,3	10,7±2,5
Diastolic events			
	Diastolic T_D (ms)	1500±300	4900±1600
	Max Diastolic Speed D ($cm \cdot s^{-1}$)	23±4	46±8
	Diastolic AUC (cm)	19±5	92±22

Systolic AUC P/ST = 1.62, p=0.08(n.s.), Diastolic AUC P/ST = 4.84, p=0.009** Diastolic TD P/ ST = 3.27, p=0.07 n.s.

Table V - Analysis of the Paraná Manoeuvre and Squeezing Test in 11 Re-Entry Perforating veins.
T_S systolic ejection time, T_D diastolic refluxing time, AUC area under the curve, P Paraná, ST Squeezing Test. Mean ± standard error of the mean.

As an easier detectable reflux provides a more reliable DUS as well as plethysmography, the quantitative advantages of P could be of paramount clinical importance in improving diagnostics in venous diseases.

In addition, P does not rely on the size of the operator's hand or the size of the patient's calf. In this paper we compared P to a manually performed ST, as this is the modality ST is generally performed in almost all vascular labs, while the standardized pneumatic compression by means of a cuff is rarely executed, being also time consuming.

Furthermore, as posture adjustments are spontaneously performed daily and in countless ways, P provides invaluable information about a condition which is almost near to the physiological posture balance. On the contrary, ST is based on external compression and relaxation, which are completely artificial operations.

This paper could also serve to encourage and to give more recognition to diagnostic manoeuvres which explore better physiological conditions.

Conclusions

In competent popliteal veins P moved 40% more blood volume than ST. The diastolic phase of P compared to ST lasted more than 3 times longer in the incompetent SFJ, more than 2 times longer in the incompetent GSV trunk, and more than 3 times longer in the re-entry Perforating veins. No significant difference in the standard deviation of the acceleration was detected between P and ST.

ST is widely practiced in most vascular labs, though nowadays several old and new manoeuvres have shown that ST is not the best choice to elicit the venous reflux[8-12]. In addition, P is undoubtedly an easy and safe manoeuvre, causing physiological changes which occur in balance adjustments in the standing position. P is actually practised since 20 y in many vascular labs and we hope that our results will support an increased utilization in the assessment of the function of the venous system.

Systolic acceleration

Acceleration Unit: $cm \cdot s^{-2}$	Average (two-tailed paired T test)			σ (two-tailed F test)		
	p	Squeezing	Paraná	p	Squeezing	Paraná
Competent Popliteal veins	0,014*	0.19	0.13	0.8 (n.s.)	0.06	0.07
Incompetent SFJ	0.008**	0.07	0.03	0.0002***	0.04	0.01
Incompetent GSV	0.21 (n.s.)	0.06	0.11	$1 \cdot 10^{-7}$ ***	0.02	0.14
Re-Entry Perforators	0.08 (n.s.)	0.06	0.04	0.10 (n.s.)	0.04	0.02

Table VI - Analysis of the standard deviation of systolic acceleration.

SFJ saphenous femoral junction, GSV greater saphenous vein, p statistical significance, σ standard deviation.

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Author contributions

Conception and design: SE, Data collection: SE, Statistics: FP, Literature review: MC, Writing the article: SE FP, Critical revision of the article: SE FP MC CF, Supervision: CF.

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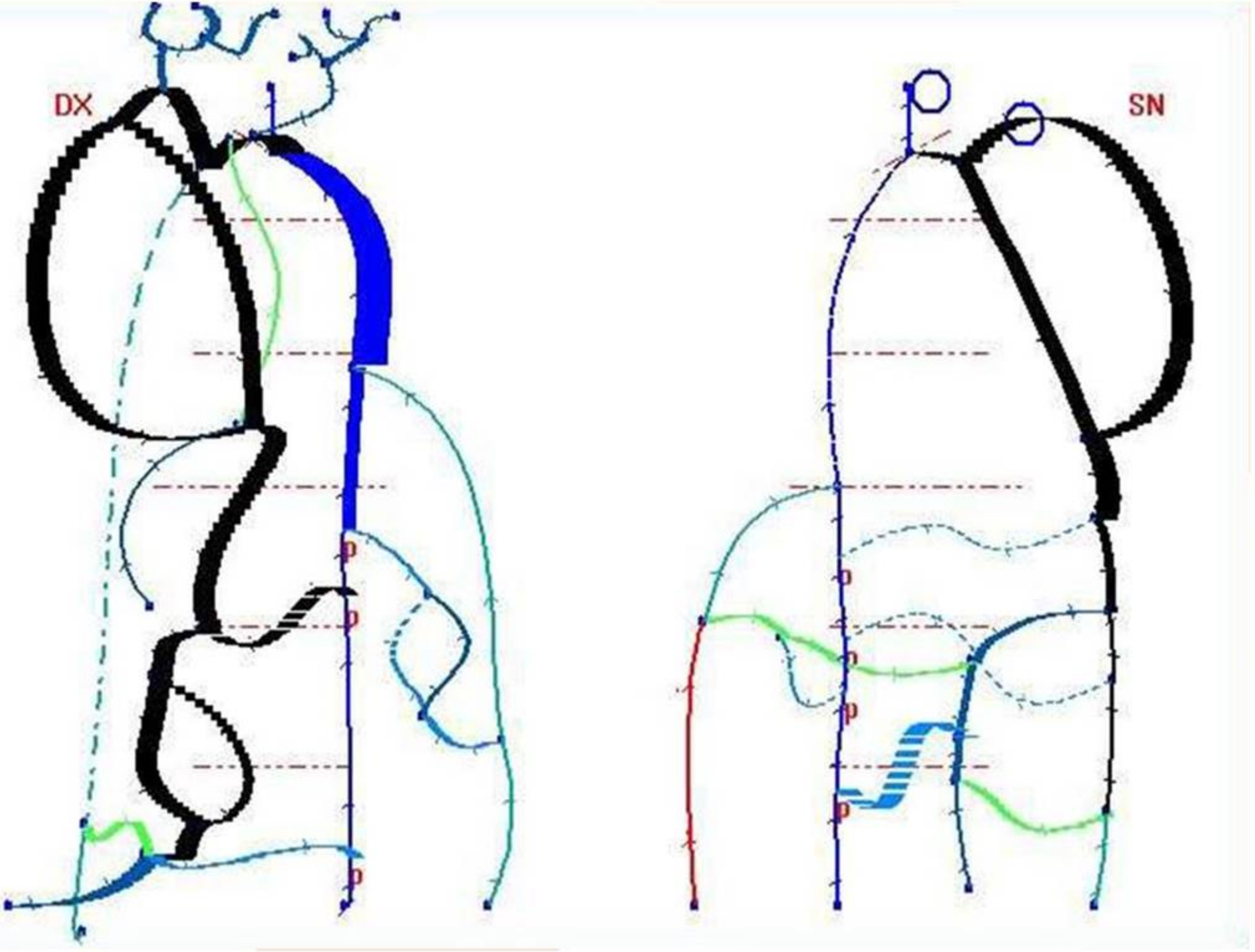
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