The greater Inflammatory Pathway – High clinical potential by innovative predictive, preventive and personalized medical Approach

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Abstract

Background & limitations Impaired wound healing (WH) and chronic inflammation are hallmarks of non-communicable diseases (NCDs). However, despite WH being a recognized player in NCDs, mainstream therapies focus on (un)targeted damping of the inflammatory response, leaving WH largely unaddressed, owing to three main factors. The first is the complexity of the pathway that links inflammation and wound healing; the second is the dual nature, local and systemic, of WH; the third is the limited acknowledgement of genetic and contingent causes that disrupt physiologic progression of WH.

Proposed approach Here, in the frame of Personalized, Predictive, Preventive Medicine (PPPM), we integrate and revisit current literature to offer a novel systemic view on the cues that can impact on the fate (acute or chronic inflammation) of WH, beyond the compartmentalization of medical disciplines and with the support of advanced computational biology.

Conclusions This shall open to a broader understanding of the causes for WH going awry, offering new operational criteria for patients' stratification (prediction and personalization). While this may also offer improved options for targeted prevention, we will envisage new therapeutic strategies to reboot and/or boost WH, to enable its progression across its physiological phases, the first of which is a transient acute inflammatory response versus the chronic low-grade inflammation characteristic of NCDs.

Keywords: predictive, preventive and personalized medicine; wound healing; inflammation; non-communicable diseases; mechanotransduction; network science; multi-omics; neuro-immuno modulation; autonomic nervous system; genetics; epigenetics; patients stratification; individualized patient profile; risk, modifiable and preventable factors; big data analysis; machine learning; phenotyping

List of Abbreviations

ANS autonomic nervous system
AR adrenoceptor
BMI body mass index
CNS central nervous system
CRP C-reactive protein
DVC dorsal vagal complex
ECM extracellular matrix
EMT epithelial mesenchymal transition
ENS enteric nervous system
ESWT extracorporeal shock wave
FMT fecal microbiota transplantation
GBA gut brain axis
GI gut intestinal
GWAS genome wide association studies
HPA hypothalamus-pituitary-adrenal
LC locus coeruleus
NCD non communicable disease
NTS nucleus tractus solitarii
PPPM predictive, preventive and personalized medicine
PRS Polygenic Risk Scores
PVN paraventricular nuclei
RA rheumatoid arthritis
RVLM rostroventrolateral medulla
SBML systems biology markup language
SNS sympathetic nervous system
TNF tumor necrosis factor
WH wound healing
WHO world health organization

1. Introduction

PPPM is concerned with the implementation of predictive, preventive and personalized approaches to medicine to grant a novel, more efficient and effective return to health or a more human control of disease, with attention to all aspects and stakeholders of the complex faces that define health, and with the very urgent mission to move away for the current reactive medical paradigm, with all means that can enhance and improve prevention. This can be applied to all realms of medicine, yet, while acute manifestations of diseases are better managed, chronicity represents a tremendous economic, social, ethical and medical burden for society as a whole.

Impaired WH and chronic inflammation are hallmarks of the majority of NCDs. Numbers recommends to carefully assess any improvement to be done in this context, as over $25 billions are spent annually on chronic wound, affecting 6.5 million patients [1,2]. NCDs kill 41 million people, between the ages of 30 and 69, each year, equivalent to 71% of all deaths globally (WHO factsheet https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases, and [3]). And although tobacco use, physical inactivity, the harmful use of alcohol and unhealthy diets are al known to increase the risk of dying from a NCD, major expenditure are still dedicated to mainstream therapies consisting mostly of controlling inflammation by targeted/untargeted damping of the inflammatory response. This focus on chronic inflammation entails (or is the effect of) several limitations. Recent work in the context of PPPM focusing on multiprofessional approaches to WH represents an innovative and needed approach to overcome these limitations [1,4,5], the current work digs deeper into the basic molecular mechanisms relevant to this issue, with an original focus on the aforementioned limitations, detailed below.

First, from a therapeutic point of view, impaired wound healing is often considered an ancillary and concomitant event to chronic inflammation, despite chronic inflammation being also a known *consequence* of WH gone awry [6,7]. This has implications in the understanding of the aetiology and progression of such diseases (unclear causality), as well as in the opportunity to address directly WH impairment. Considering WH the umbrella under which multiple players contribute to the inflammatory response could enable different approaches to perturbed WH, via other afferent/connected/overlapping functions including, remarkably, the activity of the nervous system [8,9] and mechanosensing [10,11], whose currently highly neglected advantage is that it can be activated by non-biochemical triggers (electrical and mechanical). The building bricks of this discussion are described in section 2.1 *Wound healing and the greater inflammatory response*.

Second, WH and its progression are generally considered local to an injury and are well-studied as so, with applications promoted in the clinical domain, but limited to dermatology and orthopaedics. Both, with particular attention to scarring and burns for the former and fractures for the latter, take advantage of a broader WH pathway, namely by including in healing therapies mechanical cues, known to elicit WH [12,13]. Yet, this knowledge fails to be translated into other medical domains, where chronic inflammation and impaired WH are recognized as systemic features, with rare, although promising exceptions ([14,15]). There persists in fact a limited understanding and dissemination of the mechanisms that make the *local* WH response (to the injury) a *systemic* phenomenon, revisiting literature is crucial to overcome this limitation, we will address this in section 2.2 *Wound healing, linking the local with the systemic phenomenon.*

Third, little is known about the individual genetic and contingent factors that disrupt the physiologic progression of WH, impairing its ability to resolve local inflammation. Touching briefly on the causes for healing disturbance [16], we will focus on reviewing genomic approaches to inflammation, the early phase of WH [17,18], addressing the

contrast between acute and chronic inflammation in autoimmune disease in section 2.3 *The genetics of inflammation in NCDs.*

2. Revisiting wound healing

2.1 Wound healing and the greater inflammatory response

Wound healing is a multifaceted phenomenon, known, according to the literature, to progress across three to four major phases and namely: (haemostasis), transient acute inflammation, proliferation/repair and remodeling [13], likely to be better understood when framed under the broader concept of epithelial-mesenchymal transition (EMT). EMT defines the reversible transformation of epithelial into mesenchymal cells, occurring in events apparently as diverse as embryonic cells differentiation (EMT Type1), wound healing (EMT Type2) and metastases (EMT type 3) (for a detailed description of the phenomena we refer the readers to a series or well curated articles [19–21]). Under the polyhedric light of EMT, it is easier to understand how manipulations of this function has a tremendous potential for application in medicine, in terms of regeneration (Type1), healing (Type2) and even cancer management (Type3), yet the complexity of the phenomenon has led so far to limited clinical exploitation. There is in particular a fundamental gap in the understanding of the hierarchy of systems that are involved in WH. EMT Type2 is a well understood cellular phenomenon, yet response to an injury, implies communication not only among heterogeneous cells (fibroblasts, keratinocytes to name a few), but also, importantly, with the hosting structure, i.e. the extracellular matrix (ECM), collector for numerous signals and systems. Although not always explicitly declared in the WH literature, this strongly ties WH for its role in the *local* repair of a wound to very diverse functions that include (in addition to inflammation and immunity that will not be discussed here) mechanotransduction, the response of the autonomic and central nervous system to inflammation and the gut-brain axis. The two latter, in particular, make WH a *systemic* phenomenon. We argue that the number, diversity and complexity of the functions involved, the compartmentalization of the academic areas where these functions are traditionally studied, and the limited acknowledgement of the local-to-systemic character of WH hamper our understanding and limit our possibilities to intervene in WH gone awry. For this we briefly recall here the major characteristics of these concepts, too often neglected as companions of WH.

Mechanotransduction is the biochemical response of the cell to mechanical stimuli, resulting in cellular adaptions to mechanical forces. Mechanotransduction also progresses through a number of phases. In the first few seconds after wounding, non-transcriptional signaling (i.e. those mediated by $Ca^{(+2)}$) supported by increased cell membrane permeability is observed [22]. This is followed by integrin-dependent processes and deformation of gap junctions and by transcriptional activation of secondary messenger enabling communications among cells with similar and different phenotypes, with consequences on the regulation of cell cycle and on the metabolism of ECM proteins. Finally, activation of hormones and

growth factors receptors complete the response to external forces that lead to changes in the tissue structure and function [23].

These events clearly overlap with the early stages of WH [22,24], yet, this has long been exploited, by direct observation, only in a limited number of medical specialties: dermatology, anatomy and surgery, where for instance medical doctors have observed the relevance of mechanical tension, due to the presence or absence of bones stressing the scar, on WH outcomes [25][26]. Further, physical therapeutic intervention (mechanical stimulation) on osteoarthritis, anterior cruciate ligament reconstruction and total knee arthroplasty have shown improved results [27] globally inspiring innovative therapies to address scar-less WH [28]. Along these lines, application of external energy to promote WH has been used via low-energy extracorporeal shock wave (ESWT), reported to enhance the production of vascular endothelial growth factor [29], the recruiting of skin fibroblasts, to modulate leucocytes infiltration and early proinflammatory immune response in severe cutaneous burn injury [30] [31]. The exposure of macrophage to ESWT promotes the acquisition of an anti-inflammatory profile [32], and the induction of proliferation, differentiation and immunomodulation of mesenchymal stem cells [33].

In a number of studies, application of tension on tissue appears to have positive effects on local WH, suggesting that ESWT therapies, electromagnetic stimulation and low intensity vibrations are treatments that promote healing through mechanotransduction [13] [34].

Although cellular mechanosensitivity in the healing tissue repair/regeneration process is exploited in physiotherapy, the link between biomechanism of movement and cell and tissue adaptation is still not well defined [13][35][23]. Moreover, inter-individual variability in response (towards healing or chronicity) needs to be acknowledged, with tools yet to be standardized.

The autonomic nervous system (ANS) has relatively recently become a renown additional regulator of the inflammatory and immune response. Recent advances at the intersection between immunology and neuroscience reveal reflex neural circuit mechanisms regulating innate and adaptive immunity.

The *inflammatory reflex* is a well-characterized circuit reflex [8]. It consists of afferent and efferent signals that, travelling along the vagus nerve (parasympathetic) and sympathetic nerves, results in the inhibition of the release of the inflammatory mediators and cytokines, such as tumor necrosis factor (TNF), from monocytes and macrophages.

Ample literature supports the pivotal role of the ANS and its neurotransmitters in the regulation of inflammatory response. In acute and chronic inflammation the autonomic modulation showed a sympathetic interference in the earlier stages of the inflammatory process and activation of the inflammatory reflex that regulates the innate immune responses and cytokine activity in longer processes [9]. A closer look at the phenomenon, makes its role crucial in the local-to-systemic nature of WH: when an antigen enters or a wound is perceived, the first effect is the activation of the innate immune cells that release proinflammatory mediators such as cytokines, pivotal in the communication from the immune to the central nervous system (CNS) [36][37].

Vagal and somatic sensory afferent nerve fibers detect the local inflammation trough receptors for inflammatory mediators, like cytokines or toll like receptors [38–41]. Sensory challenge by inflammatory mediators can either activate afferent signaling pathways and stimulate a local response, based on the antidromic release of neuromodulators (neuropeptides Substance P, calcitonin-gene related peptide, among others) and neurotransmitters [42–45] that have demonstrated a net anti-inflammatory outcome [9]. ANS activation, following the detection of inflammatory signals by sensory nerves, can then influence the immune systems directly, via neurotransmitters and/or neuropeptides [46] challenging their receptors exposed on immune cells surface, or indirectly, via regulation of the blood or lymph flow, modulating the distribution [47] and production [48] of lymphocytes, or influencing the release of neuropeptides (i.e. substance P) from the sensory nerve endings [49][50].

Special emphasis is placed on cholinergic anti-inflammatory mechanisms that inhibit the activation of macrophages and, although the exact signaling pathway is still matter of debate [51–53] , it is relatively clear that the neural control of acute inflammation is reflexive and potentially controllable via electrical or pharmacological activation. The original observation that vagal efferent activity stimulated by central muscarinic challenge improved the symptoms of local and systemic inflammation [54,55], pointed at the vagus as an essential effector in the neuromodulation of inflammation. The nicotinic α7nAChR, expressed on both immune cells and on sympathetic post-ganglionic neurons, was then identified as the peripheral transducer of the vagal cholinergic anti-inflammatory action [56,57]. Circulating T-cells expressing the enzyme choline acetyl-transferase (ChAT) and synthesizing ACh were also identified as non-neural link in the cholinergic inflammatory pathway [58], resolving the apparent paradox of a lack of direct vagal innervation of the spleen [59], that was indeed indicated as essential in the vagus-to-inflammation circuitry [60]. Finally, the importance of sympathetic noradrenergic innervation of the spleen and/or the peripheral site of inflammation and the role of β2-AR in mediating the anti-inflammatory sympathetic action, has been elucidated [53,61–63]. Based on the increasing knowledge about the mechanism(s) underlying the neuro-immune crosstalk after the establishment of inflammatory/reparative processes, new therapeutic modalities have been proposed and tested in preclinical and clinical settings. Indeed, experimental activation of the cholinergic anti-inflammatory pathway by direct electrical stimulation or pharmacological means of the efferent vagus nerve prevents inflammation and inhibits the release of cytokines that are clinically relevant drug targets for treating inflammatory disease in liver, spleen and heart, and attenuates serum concentrations of TNF during endotoxaemia [64,65]. Applications of these findings have a poorly exploited therapeutic potential that will be discussed in Section 3.2.

The gut brain axis (GBA) is a complex interaction between brain and gut, enabling the interconnection between the cognitive and emotional brain centers with the intestinal function in relation to immune activation, enteric reflex and entero-endocrine signaling.

The enteric microbiota has a pivotal role in the GBA, with the ability to produce systemic effects via neuroendocrine and metabolic pathways making possible a direct interaction with

247 the CNS and with the enteric nervous system (ENS). Local effects also use the same
248 metabolic and neuroendocrine pathways directly on local intestinal [66]. Via the GBA, the
249 CNS, the gut-intestinal microbiota (see below) and the immune system are implicated in the
250 etiopathogenesis or manifestation of neurodevelopmental, psychiatric and neurodegenerative
251 diseases, such as autism spectrum disorders, depression and Alzheimer's disease [67,68],
252 opening to completely new approach to these diseases, including fecal microbiota
253 transplantation (FMT) [69].
254
255 **The gut-intestinal (GI) microbiota** represents the complex ensemble of microbes that live
256 in synergy with us, and in particular that are located in the distal part of the large intestine,
257 constituting the better known and larger community. It is now well assessed how the GI
258 microbiota is relevant in the etiology of NCDs regularly accompanied by dysbiosis [70–74].
259 Its connection via the ENS to the GBA is obvious and bidirectional, as in turn GBA
260 demonstrate a critical role for the gut microbiota in orchestrating brain development and
261 behavior, and the immune system is emerging as an important regulator of these interactions.
262 Similarly the correlation between dysbiosis (non-physiologic composition of the gut
263 microbiota) and NCDs is also clear [75–78].

264 **2.2. Wound healing: linking the local with the systemic phenomenon**

265 Wound healing and the process of tissue repair require a complex and finely regulated
266 feedback and feed-forward interaction between the immune and the nervous system.
267 Among the 4 stages of WH (haemostasis, inflammation, proliferation and remodelling),
268 inflammation is critical for the removal of the primary trigger and to promote the
269 progression of WH toward tissue repair [79]. In a physiological framework, acute
270 inflammation is essential for a restorative response, is self-limiting, and followed by tissue
271 formation and remodelling [80]. The fine-tuning of inflammation is then a critical need in
272 the process of WH, the completion of inflammatory stage being the crossroads between
273 healing or establishing chronic pathological conditions.

274 Local and systemic inflammation are controlled and modulated by the interaction of
275 nervous and immune system, in a complex crosstalk mechanism that has been referred as
276 neuro-immunomodulation (recently and extensively reviewed in [81,82]) schematically
277 represented in Figure 1. Such physiological control system aims at maintaining immune
278 homeostasis and avoiding excessive immune over-activation. Interestingly, dysregulated
279 inflammation with impaired WH, are described in several physio-pathological conditions -
280 such as ageing, malnutrition, diabetes, vascular insufficiency - characterized by deficiencies
281 in nervous system function, resulting in ineffective neuromodulation of the immune
282 response [83].

283 The functional neural circuitry operating in the control of inflammation works according to
284 the classic homeostatic paradigm [84]. This requires an afferent component, *sensing* the
285 inflammatory state and an efferent arm, which is the effector generating the

immunomodulatory signal at the site of inflammation. In between, the circuit includes a control centre, whose role is to process multisensory inputs, integrating them with cognitive functions and the needs for proper adaptive behavioural responses before activating the efferent arm [82]. The first evidence of such a regulatory mechanism operating in the control of inflammation [54,55] led to the definition of the classical *inflammatory reflex* [8]. Sensing inflammation is the first step toward the activation of a proper neural control of WH. As briefly recalled in Section 2.1, two types of sensory neurons convey relevant information about local and systemic inflammation to the integrative centres in the spinal cord and the brain: somatic sensory neurons, with cell bodies in the dorsal root ganglia (DRG) and vagal afferent neurons, having cell bodies in the nodose and jugular ganglia [81]. Somatic afferent signals travel through the spinal cord, in multi-synaptic pathways, toward their integrative nuclei located in the thalamus and brainstem, finally reaching limbic and cortical targets. Vagal afferent signals are mainly directed toward the nucleus tractus solitarii (NTS) in the brainstem. The main differences, between the two sensing systems, resides in the type of inflammatory stimuli that generate their activity. Indeed, somatic afferents are mostly conveying information about inflammation at the body surface or in the musculoskeletal system, while vagal afferent signals are generated by inflammation of visceral organs or the whole biological system (systemic inflammation).

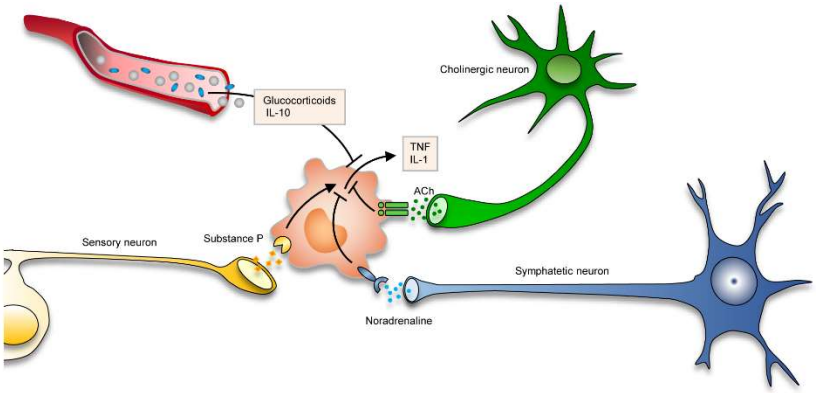

Figure 1. Different effector pathways controlling inflammation are coordinated by brain activity. Circulation delivers inflammatory cells and diffusible factors (such as cytokines and anti-inflammatory hormones) to and from the inflammatory site, establishing slow and concentration gradient-dependent anti-inflammatory response. The local, fast neural anti-inflammatory regulation is exerted by cholinergic and noradrenergic neurons, releasing their neurotransmitters and predominately inhibiting pro-inflammatory cytokines release from immune cells. Sensory neurons are instead effective in stimulating cytokine synthesis and release, amplifying the local inflammatory response.

The efferent arm of the nervous system modulating inflammatory response, its anatomic and functional organization, the identification and characterization of molecular mediators and pathways activated and the overall evolution of scientific knowledge of the matter has been extensively reviewed in the last few years [53,59,61,81,82,85–88]. Though still under investigation, the complexity of the efferent neural circuits capable of modulating and

hampering inflammation has been mostly unravelled and actually, the importance of both parasympathetic cholinergic and sympathetic catecholaminergic systems have been recognized. The peculiar feature that deserves attention is that the two efferent branches of the autonomic nervous system, classically described as antagonistic, may work in convergent or in sequential mode, when challenged to dampen inflammation [53,82,89]. The recruitment of vagal and/or sympathetic response may depend on the site of inflammation, the individual physio-pathological state, the characteristic of the inflammatory signals conveyed to the central nervous system and the different central modalities activated in response to different sensory inputs.

Central processing of afferent inflammatory signals and their integration with multisensory inputs as well as with higher affective and cognitive instances, is a still under-explored issue, representing the next challenge in the need for understanding neuro-modulatory mechanisms [82]. Three effector pathways controlling inflammation are (simultaneously) coordinated by brain activity in response to sensory signals: the hypothalamus-pituitary-adrenal (HPA) axis, the parasympathetic and the sympathetic nervous system. HPA provide a long-lasting, humoral (slow) response through the blood stream, based on the final release of glucocorticoids from the adrenal cortex. The SNS provide a mix of humoral and fast-acting neural response, played by catecholamines released both locally in the organs and by the adrenal medulla in the bloodstream. The parasympathetic nervous system provides a pure neuronal response, mediated by Ach and characterized by local and transient effects.

Sensory signals generated by somatic afferents, travel through the spinal cord to the thalamus and the brainstem rostroventrolateral medulla (RVLM) and locus coeruleus (LC) [90]. Their central processing and integration with other brain functions then, take place in the somatosensory cortex, and the limbic system. Vagal sensory signals are directed toward the NTS and then transmitted both to adjacent vagal nuclei encompassing the dorsal vagal complex (DVC) and to the hypothalamus, cortex and forebrain nuclei [91]. All of these brain nuclei are interconnected in multi-synaptic circuitries. Attempting to generate a map of brain nuclei activation during systemic inflammation, *c-fos* expression was studied after intestinal infection in rodents [92], demonstrating a substantial activation of NTS, area prostrema, RLVM, LC, thalamus, hypothalamus, amygdala and insular cortex. This gives the idea of the complexity of potential brain networks participating in the regulation of efferent neural anti-inflammatory pathways. It is worth noting that, among the well described central descending control systems of vagal and sympathetic activity, a special emphasis has been recently put forward on the activation by vagal afferent stimulation of brain sympathetic excitatory nuclei, namely the LC and paraventricular nuclei (PVN) of the hypothalamus, improving joint inflammation in a model of arthritis, in a β-AR-dependent way [93,94].

Several central neurotransmitter systems have been investigated for their role in the modulation of the inflammatory response. Acetylcholine, through muscarinic signalling, has been the first central neurotransmitter implicated in controlling peripheral inflammation through the inflammatory reflex and the suppression of serum TNF [55]. Basal forebrain

cholinergic neurons, described as modulators of learning and memory functions, when activated may suppress serum TNF in a murine model of endotoxemia [95]. Stimulation of tyrosine hydroxylase-expressing brainstem RLVM neurons protects against aberrant inflammation of internal organs, an effect depending on both sympathetic and vagal integrity [96]. Dopaminergic signalling in the ventral tegmental area of the midbrain, a central reward regulatory system, has also been highlighted as a possible player in the regulation of immune functions, peripherally mediated by sympathetic catecholaminergic neurons [97]. Beside pointing at the enormous complexity of the central neural networks potentially involved in the regulation of immune functions, these evidences indicate that emerging therapies based on brain stimulation methodologies for the treatment of neurological diseases (i.e.: transcranial magnetic stimulation, deep brain stimulation, transcranial direct-current stimulation) may be also useful as immune-modulatory therapies [82].

2.3. The genetics of inflammation in NCDs

There are essentially two approaches to the genetics of inflammation as it relates to NCDs. One is to characterize the regulation and activity of individual components that mediate inflammation, and the other is to consider inflammation as a complex trait captured by a biomarker such as C-Reactive Protein (CRP). Since the vast majority of associations identified by GWAS are due to regulatory polymorphisms, it is no surprise that there is pervasive genetic variation influencing the expression of key components of key inflammatory mediators such as the inflammasome, or inflammatory macrophages and microglia. Many of these are also associated with inflammatory autoimmune or other chronic diseases including coronary artery disease, type 2 diabetes, and Alzheimer's disease. Examples too numerous to review here include interferons, interleukins and other cytokines, pattern recognition receptors, receptors and ligands involved in T-cell exhaustion, and extracellular matrix components, as reviewed by [98]. Epigenetic regulation is also commonly observed, and research is beginning to reveal how the microbiome, nutritional, and psychosocial stress influence their regulation.

Concerning systemic inflammation, genetic studies have been most revealing for chronic levels of CRP, and it is unfortunate that insufficient attention has been given to the induction of the inflammatory response upon infection or wounding. To our knowledge, the largest genetic study of chronic inflammation to date published in late 2018 [17] was a genome-wide association study of circulating CRP levels. Analysis of over 200,000 European-ancestry individuals sampled in 88 studies around the world identified 58 distinct loci collectively explaining up to 11% of the variance in CRP, with similar effects in both sexes, for the most part independent of body mass index. The largest effects were observed at the *CRP* locus itself (where a total of 13 independent signals were documented) and at various well-known inflammation mediators including *IL6* and its receptor *IL6R*, and the *APOE*/*APOC1* locus. Pathway analysis implicated numerous gene sets involved in immunity and metabolism and found enrichment for gene expression in many different cell types, all consistent with the systemic and complex nature of inflammatory regulation. Importantly, Mendelian

randomization analyses found evidence that CRP is protective against schizophrenia but causal for bipolar disorder, yet found no evidence for causality in relation to coronary artery disease, Alzheimer's disease, Crohn's disease or rheumatoid arthritis (RA). That is to say, the evidence is more consistent with genetics playing a role in the capacity of CRP to resolve or promote inflammation that accompanies these NCDs than in promoting them. Another large GWAS for CRP incorporating Mendelian randomization [18] found some evidence for causality in Type 2 Diabetes, but not Type 1 Diabetes, confirming an inflammatory contribution to the now more common form of the disease.

2.4. The epigenetics of inflammation in NCDs

Given the suspicion that epigenetics may also play a role in inflammation, large genomic studies have also considered the relationship between methylation and CRP. A sizeable study of peripheral blood samples from Crohn's disease paediatric patients at initial diagnosis identified almost 1,200 CpG sites that were differentially methylated relative to healthy controls, but by one year of follow-up the signature had virtually disappeared, irrespective of disease status [99]. Further investigation revealed that the differential methylation was very highly correlated with the association of CpG to CRP levels [100], implying that inflammation accompanies onset of disease and leads to epigenetic modification of the DNA in immune cells that recedes with time. Mendelian randomization analysis again found little evidence for a causal role for the inflammation-associated methylation in pathogenesis, instead suggesting that altered methylation is responsive to inflammation, a finding also reached in a very large peripheral blood epigenome-wide association study of body mass and obesity [101]. Nevertheless, many NCD-associations identified by GWAS are also associated with the expression of local transcripts or level of methylation of linked CpG [102,103], including numerous loci in inflammatory pathways, illustrating the complexity of genetic impacts on inflammatory disease.

2.5 Computational tools to revisit WH

2.5.1 Network theory and the multi-omic approach

The biological mechanisms described above clearly represent a complexity that covers different temporal (from nanoseconds for early pre-transcriptional signals, 10^{-9} s, to years for full repair, 10^{8} s) and physical (10^{-10}m for molecules to 1m for effects on the whole organism) scales [104]. The Cartesian, reductionistic approach has successfully achieved the goal to simplify our understanding of phenomena by breaking them into simpler, more homogenous subsystems (nervous, immune, genetic, etc.) that can now be described in much detail. However, this overlooks the *emergent properties,* i.e. the characteristics that are visible and open only when the system is studied in its entirety, that is when the ensemble interacts [105,106], therefore an additional effort is needed to represent and understand phenomena, in particular once complexity has become an ally rather than an enemy. This concept has been translated from engineering when systems theory was born to biology with systems biology [106] and finally to systems medicine [105], matured into PPPM [107]. This is naturally occurring, as we have shown above, with progressing discoveries: neurophysiology

integrates microbiology in the gut-brain axis and immunology and neurophysiology have an intricate communication, however further steps must be taken to further understand this complexity up to the point of knowingly manipulating (i.e. treating) the system (i.e. NCD patients).

The transdisciplinarity of biomedicine has progressed with the advent of *omics* and expanded to include novel technologies (from microarray to next generation sequencing, NGS), novel molecular data (epigenomics from miRNA-seq to methylomics, now including single cell and spatial RNA-seq, and microbial metagenomics) and the synergy with exact sciences has now evolved into *computational biology*, with the introduction and application to medicine of sophisticated approaches. While machine learning (ML), and deep learning in particular, is enabling tremendous progresses in the automation of complex clinical tasks and in (molecular) pattern discoveries, network approaches are the ideal tool to handle representations of complexity, offering, in some of their implementation, suggestions as to causal links [108,109]. Notwithstanding the advanced mathematics that can be involved in the analysis, the starting, descriptive point is extremely intuitive, as it boils networks down to a couple of concepts: i) a set of nodes (any entity) and ii) a set of edges (any relationship among entities). Nodes allow visual representation of heterogenous entities (proteins, genes, transcripts, metabolites) and their interactions (phosphorylation, activation, docking, etc.) no matter the complexity of the reticulum they form, thus naturally enabling heterogeneous data integration and in particular multi-omics (genomics, transcriptomics, epigenomics, proteomics, etc. [110,111]). Additional concepts can be introduced to describe the flow of information from nodes across edges: from tokens [112] to (binary) activation [113], to probability priors [114], only to name a few. Network can then well represent pathway and *dynamic* and *topological* analyses promoted by *in silico* experiments.

D*ynamic* analysis enables simulations of *what-if* scenarios describing the short, mid or long-term effects of the perturbation of the network [115–119], that, depending on the network type in use, can represent changes in the abundance of a molecule (including lack, i.e. malfunctioning of a molecule, a.k.a. deletion of the node) or modifications in the connection between nodes (including lack, i.e. absence of signaling a.k.a. interruption of the communication; or new edges, i.e. alternative pathways).

T*opological* analysis enables us to rank and formalize the relevance of (groups of) molecules as *key* or *ancillary* to the proper functioning of the whole network (pathway) with a focus on the communication flow (signaling). Among the hottest topics for research in this context is the identification of *communities* (sets, clusters, group, i.e. nodes/molecules/microbes with a more similar behaviour among the group member than with the rest of the network [120–123]). Communities can often be recognized as surrogates for biological functions. Further, the concept of *centrality* i.e. the extent to which a node is an intermediate in communication (signalling) -computed with a variety of definitions [124]- enables the quantified ranking of potential proxies of therapeutic targets [125]. Starting from the intuitive *hub*s (i.e. the nodes presenting the highest number of edges in the network, i.e. the most connected molecule in the pathway) moving to energy-based or probabilistic approaches [126] it is possible to

achieve a more sophisticated description of the relevance of a node in the economy of the network communication, identifying nodes/molecules that mostly support the efficiency of the communication/signalling. Ranking by centrality can offer the opportunity to identify alternate key molecules, shall the top ones be unavailable (genetics, environment, drugs) highlighting the creation of secondary communities or pathways, that can be correlated to side or adverse effect.

Current limitations to this approach are many-fold. On one side, only a part of the classical pathways exist in the form of networks in the popular, highly curated databases, like the ones drawn by CellDesigner using SBML (Systems Biology Markup Language, the proposed *lingua franca* for systems biology [127]). There is a lack for example of public mechanotransduction pathways [128], only partial representations of WH/EMT [129,130], and limited network description of the host-microbiome interface [131,132]. Certainly, the state-of-the art complexity described in the sections above has not yet been translated into networks.

Overall, the practical and direct output of the creation of such an integrated network would be a redesign of the topology (molecules from more pathways and differently wired [133–135]) of the inflammatory process, with different central nodes that are surrogates for key molecules and potential biomarkers, and/or different communities (surrogates for functions). An example of such a topological reorganization is shown in Figure 2. This will for example make very obvious the relevance of mechanotransduction, whose early activity fully overlaps with the early phases of WH/EMT Type2, and can make explicit the connection between mechanotransduction and the nervous response to inflammation, rarely discussed in literature [136,137]) nor translated into medicine. Smaller scale approaches have already suggested the potential for integrins as drug targets [10,11].

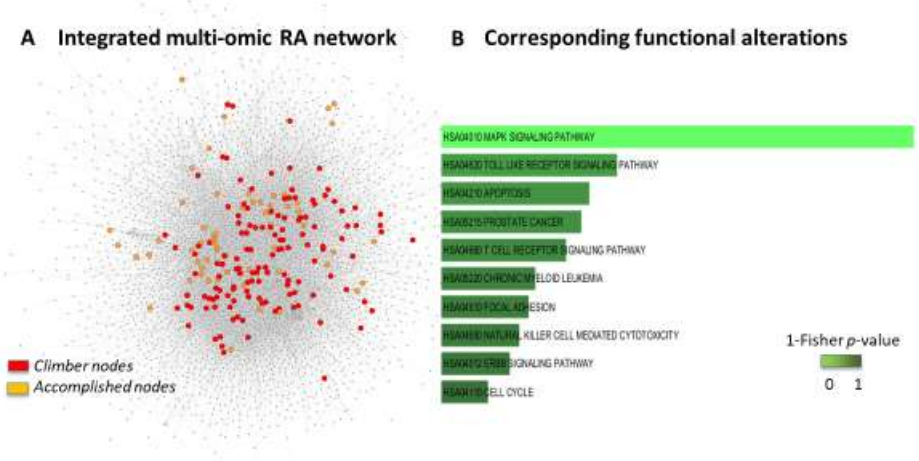

Figure 2. Adapted with permission from [132]. The integration of multiomic information to represent RA molecular network. Panel A shows the density of the new multi-omic integrated network (grey nodes) versus the original transcriptomic network (red and orange nodes). Red and orange nodes are classified based on their topological characteristics (number of edges, connectivity) as climbers if the number of edges increases after integration in the new network, or accomplished if the number is stable. Panel B shows the same information at the functional level (i.e. which functions are altered by modifying the topology). This operation joined to biomedical considerations enabled the identification of IRK4 as a relevant molecule with potential side and adverse effects.

2.5.2. Big data and machine learning approaches

The identified molecules can seed additional approaches *in silico*, before entering costly clinical trials, thanks to the large and increasing production of big data (personal, economic, social, environmental and clinical records, representing 10^{18} bytes in the United States and growing 48% annually [138]) more and more often coupled with associated biobanks and *omic* data (Twins UK https://twinsuk.ac.uk/, Swedish twins registry https://ki.se/en/research/the-swedish-twin-registry, Center for Health Discovery and Wellbeing Cohort https://predictivehealth.emory.edu/research/resources.html). Examples of short to mid-term projects include interrogation of such databases in search of clusters/signatures and other more complex patterns built around the most promising key molecules identified by *the greater inflammatory pathway* (with some of the basic artificial intelligence (AI) algorithm and in particular supervised ML algorithms [139,140]). These in turn can provide novel molecular surrogates for better patients' stratification, better and faster therapy definition, higher success rate in disease remission. Further, molecular surrogates of clinical traits can serve as a Rosetta Stone to interrogate, in the absence of biobanks and molecular data, other cohorts' databases, revisiting responders, non-responders, comorbid phenotypes in the new light offered by the expanded molecular knowledge.

Finally, in the long run, such key molecules are, by definition, interesting therapeutic targets, therefore new drugs and therapies can be repurposed, designed and envisaged (network pharmacology [141,142]).

In addition to the curation and analysis costs, other factors seem to be relevant in this context, confirming the importance of transdisciplinary teams in biomedicine-related areas. In fact, the introduction of the potential of mechanotransduction in medicine is extremely difficult, likely hampered by a cultural bias against non-biochemical therapies, exemplified by the extremely limited, although successful, research in this direction [15,34,143]. Indeed, not only computational biomedicine is needed to overcome the current paradigm, but very likely the cooperation with anthropologists, sociologists and psychologists to elucidate both the root of the diffidence towards mechanical cues as biochemical triggers, and the broader perception of such a therapy on patients, in particular, in Europe and the USA: this global approach is indeed the among the aims of PPPM [107].

3. Perspectives in PPPM Medicine

The information integrated above, and to the best of our knowledge for the first time in such a unified and interdisciplinary scheme, enable us to envisage new responses to the major requests of PPPM, ~~and~~ namely: (i) criteria for individualized (genetics, environmental) diagnosis, representing potential new operational criteria for patients' stratification; (ii) targeted preventive measures may descend from the criteria identified in (i), for example on individuals genetically susceptible or having been exposed to environmental stimuli known to be associated to WH and inflammation; (iii) innovative therapeutic strategies to reboot and/or boost WH. Clearly, only the factual computational integration suggested above and the completion of experimental and clinical research will provide conclusive evidence, however, with this article we want to point the attention to the relatively little effort needed to move a big leap forward in our understanding of WH, i.e. we want to highlight how far we can already go once the artificial barriers of clinical and biological specialties can be transformed into a cooperative effort, once computational approaches exploit complexity rather than approximating it to its nearest simplification.

3.1 Individualized patients' profile and targeted preventive measures – environment and genetics

At the current level of understanding of the greater inflammatory pathway, better stratification must become the first objective, which, once omics are made available patient-wise, can reach the extreme point of stratification i.e. individualized treatment.

With the proposed rationale for stratification, individualized prevention becomes also an objective at reach, confirming the crucial need to make the integration described above effective, in order to achieve the deeply intertwined objectives of PPPM, i.e. patient stratification first to reach individualized patient profile then, as well as risk, modifiable and preventable factors identification. Owing to the complexity of WH and to the compartmentalized literature, environmental factors associated to WH are rarely reviewed in a systematic manner, with few exceptions [1,4,5]. Integrating from there, is, however, possible to collect a list of factors susceptible to impair WH, scattered across specialized literature. Biochemical factors include: high glucose levels [144], hypoxia [16], [145], pre-existing infection, macrophage activity (impaired by corticosteroids), bisphosphates, denosumab, oestrogen regulation, and hence sex [146], and biologicals [145], regulation of the matrix metalloproteinases (MMPs)/ tissue inhibitor of metalloproteinase (TIMPs) complexes [1,4,5]; other macroscopic (broadly environmental) factors include: moisture [147], oedema[145], ethanol abuse, smoking, stress, too low to too high BMI [148], omega-3 fatty acids intake and lack of vitamin A [145], aging, also owing to increasing stiffness of the ECM and consequently altered T-cells mobility [144].

Both the biochemical and macroscopic category are generally referred to their effects on *local* wound healing, i.e. collected within the dermatology and orthopaedics clinical experience. However, knowing that WH is a continuum, this information offers a relevant starting point to design questionnaires for early screening of NCDs, assessing, for example, the impact of dehydration and high sugar diets on impaired *systemic* WH.

Personalized genomic medicine spans a spectrum from precision diagnosis of congenital abnormalities to predictive health aimed at preventing onset or progression of complex disease. Next generation DNA and RNA sequencing is now being used effectively for

clinical applications with clinical diagnosis rates over one third for a wide range of birth defects [149]. These methods are not appropriate for wound healing applications where the proximate cause is an accident rather than a genetic abnormality. However, functional genomics may play a role in stratifying patients with respect to the course of disease. For example, Desai and colleagues [150] showed that longitudinal gene expression profiling of peripheral blood samples from 168 blunt force trauma patients over 28 days effectively identified five dynamic co-expression modules that differentiated subjects who succumbed to the trauma, or recovered at different rates. Interventional follow-up studies have not been forthcoming, in part due to the high expense of randomized clinical trials that would demonstrate clinical efficacy.

Transcriptomics has similar potential with respect to NCDs. The company PredictImmune is developing a blood-based RT-PCR signature of T-cell exhaustion [151] that is able to discriminate cases likely to enter remission from those requiring aggressive therapy to prevent progression for a range of inflammatory autoimmune diseases such as IBD, lupus and vasculitis. They note that almost 100% of physicians see the need for such a test that could reduce treatment costs by 30% or more. This is particularly relevant in IBD and RA where step-up therapy involves expensive anti-TNFα biologics [152], though there is some evidence that early treatment can prevent complications for penetrating Crohn's disease [153]. Furthermore, intestinal tissue from patients that have high levels of Oncostatin M and other inflammatory markers is strongly associated with resistance to anti-TNFα therapy [154]. Related data is emerging from transcriptomic and epigenetic profiling of synovial fluid of RA patients [155]. Notably, since genomic and standard histopathological criteria can be somewhat orthogonal, combination of these measures should greatly improve the sensitivity and specificity of predictive algorithms [153].

Regarding genotype-based tests, much interest has been generated in the use of Polygenic Risk Scores (PRS) to evaluate likelihood of disease. These are weighted sums of the effects of hundreds to millions of SNPs whose effect on a disease was ascertained by meta-analysis of very large GWAS. The prominent study of Khera et al. [156] showed that for coronary artery disease, atrial fibrillation, Crohn's disease, type 2 diabetes, and breast cancer, the top percentiles of PRS have lifetime risks of disease that are more than 3-fold higher than for the general population. Since risk this high due to Mendelian variants has been regarded as clinically actionable for some time, and orders of magnitude of people are at risk due to their polygenic background, use of such scores in predictive and preventative health is being advocated, despite their only explaining between 10% and 25% of the disease risk. The CRP GWAS explains this proportion of variation as well, and although a PRS was not reported in [17] it is highly likely that a CRP-PRS will soon be available that identify that fraction of the population who are genetically predisposed to either very high or very low levels of chronic inflammation.

We can imagine two types of application for such a test. One is as an adjustment variable in genetic association studies for NCDs. Just as adjustment for body mass index significantly improves the yield of genetic associations for type 2 diabetes [157], it would be interesting to know whether adjustment for systemic CRP, inferred from genotypes, can modify the genetic dissection of inflammatory NCDs in particular. The second application could be in prediction of response to anti-inflammatory medications. More understanding of the relationship between chronic CRP and acute inflammatory responses is needed, though: are people with normally high levels of CRP hyper-sensitive to a damaging inflammatory response, or protected since they are less likely to mount a synergistic systemic response? Similarly, do people with low genetic liability to CRP production require different interventions to promote wound healing, or are they particularly susceptible to abnormal inflammation? It is worth noting in this context that genetic evaluation has just as much potential for positive prediction of response to therapeutic intervention, as for negative prediction to avoid unnecessary, expensive or potentially damaging therapies [158].

3.2 The importance of phenotyping

In order to guide clinicians along the process that takes the medical approach from reactive to preventive all cues susceptible to give early signs of future WH alterations should be taken into considerations. Recent PPPM literature focussed specifically on the taxonomy of such maladies, using as exemplar Flammer [159–161], and related syndromes namely “dry mouth”, particularly relevant in youngster and hence with high potential of remission [148] and Sjögren [162] syndromes.

Other diseases constitute important prodromal or concomitant signs of WH potentially gone awry and include: diabetes mellitus [145,163], Down and Klinfelter syndromes, Ataxia-telangiectasia, disorders of haemoglobin synthesis (Sickle cell anaemia, Thalassemia), vasculopathies, Ehlers-Danlos and Progeroid syndromes such as Werner syndromes, autoimmune disease (primary antiphospholipid syndrome, systemic lupus erythematosus, rheumatoid arthritis), vascular diseases, a careful review with rationale for this taxonomy can be found in [4,5].
In addition to the diagnosis of such diseases, all observations referring to impaired wound healing (slow healing, excessive scarring etc.) represent additional cues as to the potential of WH having gone awry.

A special case is represented by cancer. As we recall in the introduction WH is also known as EMT type2, sharing with all other types of EMTs a tremendous overlap of pathways. In particular EMT type 3 corresponds to the metastatic process, very obviously indicating how alterations that may appear as minor into the global evolution of EMT can be relevant in the context of tumor development and progression [164], supporting also our idea of integrating and expanding the concept of inflammation and WH to include mechanosensing [15].

3.3 Enhanced spectrum of treatment options available

So far, various clinical trials have investigated the use and efficacy of parasympathetic neuromodulatory techniques in the treatment of inflammation (bioelectronic medicine).

Such large body of research (partially reviewed here but extensively reviewed, among others, in [82]) leads to the implementation of innovative therapies for controlling inflammation, based on bioelectronics stimulation of the vagus nerve (VNS) [82,86]. Implantable bioelectronics devices that activate the neural anti-inflammatory pathway have been tested in the clinical setting on patients affected by RA, with evidence of reduced TNF production and reversible improvement of clinical signs [14,165,166]. Chronic stimulation of the vagus nerve also induced disease remission in patients affected by Crohn's disease, which experienced improvement in biological parameters and in abdominal pain perception [167]. Sepsis [168], kidney ischemia-reperfusion injury [169], have also benefitted from such approaches. Generally, long term stimulation requires implantation of a device, which is not free from economical and psychological implications for the individual, as a more multidisciplinary approach would highlight.

Leveraging on these issues, a promising alternative to pharmacological and bioelectronics treatments relies on physical therapies based on stimulation of somatic sensory afferents. Among others (massage, local vibration therapy, local pressure, [34]) , mechanical and electrical stimulations by devices of the size of a needle (be it called acupuncture or its Western derivate electroacupuncture) have been proven free of adverse effects and effective in stimulating the neural mechanism(s) dampening excessive inflammation in specific contexts [89,170]. Needling therapies activate deep cutaneous and muscle mechanoreceptors, generating a local, segmental and central response after sensory afferent excitation [171]. The local effects are based on cellular and tissue mechanotransduction in response to needle insertion, and rely on purinergic signaling and mediators, such as adenosine, released by connective tissue fibroblasts [172,173]. The afferent signals directed toward spinal neurons, elicit a segmental response, reflexively activating the sympathetic efferent [174]. Finally, sensory signals generated by needling therapy generate complex and integrative responses in brain areas such as the hypothalamus, brainstem, limbic system and somatosensory cortex [175]. Based on this neurophysiological substrate, such stimuli have been tested in preclinical models of peripheral inflammatory diseases, with the aim to activate the beneficial neuromodulatory mechanism(s) of immune response (recently and extensively reviewed in [89]. Stimulation of the sciatic nerve by electrical needling has been proven effective in controlling systemic inflammation in mice, through mechanisms encompassing dopaminergic and sympathetic activities [176]. Electrical and manual needling also decreased the local levels of inflammatory cytokines in a model of collagen-induced arthritis and in experimental colitis [89,170,177]. Physical therapies struggle to be accepted in mainstream clinical practice, despite their potential low costs, ease of delivery and the generally beneficial involvement of the patient as a sentient and aware player in the therapeutic process [34].

Finally, having established the pivotal role for the autonomic nervous system in regulating intestinal immunity [178,179] together with the prevalence of the intestinal disturbances or

diseases that are associated with neuronal activity makes the innervation of the gut an appealing target for new treatment methods. As a consequence, dietary and nutritional interventions to alter the GI microbiome [66] should also be exploited, with far higher expectations than in current practice.

4. Conclusions and Experts Recommendations

We have highlighted the major steps of the roadmap to follow to fill a major lack in our understanding of basic phenomena underlying NDCs. First and foremost integration is needed: starting from the biological level, neurophysiologists, physiologists, microbiologists with the support of computer scientists must recollect the existing knowledge, scattered across specific literature, in specialized language to reach a universal biological pathway of inflammation, details on how to achieve this are given across Section 2. Further, with this knowledge at hand, medical doctors supported by biologists need to correlate molecular surrogates and phenotypic traits to give a new interpretation to the huge mass of data already produced and paid. This can already tremendously improve our understanding and offer rudimentary and yet crucial tools to enable more sophisticated patients' stratification, the first step towards personalization and individualized risk management and prevention, via questionnaires and other dedicated screenings as discussed in Section 3.

Finally, more work will be needed, introducing the expertise of social scientists as well as patients in order to transform this enhanced knowledge and more performant prevention also in innovative therapies, compatible with patients' involvement, compliance and ultimately better health.

In fact, to date, each of the faces of inflammation/WH, i.e mechanotransduction, SNS, GBA, have elicited interest for novel therapies and output innovative approaches, limited however to the clinical domain that gave them birth. In the integrated perspective we propose, it will be possible to revisit the output of pilot clinical trials and to integrate multiple approaches to gain enhanced or better modulated effects. Further, it is important to acknowledge that inter-individual variability in WH response rates exist and that causes needs to be elucidated in order to efficiently enable PPPM. None of these approaches is likely to be resolutive per se, yet, the personalized and knowledgeable integration of different forms of stimulation of the systemic wound healing process is granted to achieve better results in NCDs than we are expecting so far.

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