Nrf2 in early vascular ageing: calcification, senescence and therapy

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Abstract

Under normal physiological conditions, free radical generation and antioxidant defences are balanced, and reactive oxygen species (ROS) usually act as secondary messengers in a plethora of biological processes. However, when this balance is impaired, oxidative stress develops due to imbalanced redox homeostasis resulting in cellular damage. Oxidative stress is now recognized as a trigger of cellular senescence, which is associated with multiple chronic 'burden of lifestyle' diseases, including atherosclerosis, type-2 diabetes, chronic kidney disease and vascular calcification; all of which possess signs of early vascular ageing.

Nuclear factor erythroid 2-related factor 2 (Nrf2), termed the master regulator of antioxidant responses, is a transcription factor found to be frequently dysregulated in conditions characterized by oxidative stress and inflammation. Recent evidence suggests that activation of Nrf2 may be beneficial in protecting against vascular senescence and calcification. Both natural and synthetic Nrf2 agonists have been introduced as promising drug classes in different phases of clinical trials. However, overexpression of the Nrf2 pathway has also been linked to tumorigenesis, which highlights the requirement for further understanding of pathways involving Nrf2 activity, especially in the context of cellular senescence and vascular calcification.

Therefore, comprehensive translational pre-clinical and clinical studies addressing the targeting capabilities of Nrf2 agonists are urgently required. The present review discusses the impact of Nrf2 in senescence and calcification in early vascular ageing, with focus on the potential clinical implications of Nrf2 agonists and non-pharmacological Nrf2 therapeutics.

Key words: oxidative stress; Nrf2; early vascular ageing; cellular senescence; calcification

1. Introduction

1.1. The Nrf2 signaling pathway

Redox homeostasis, comprising a balance between a pro-oxidative reactive oxygen species (ROS) production and concomitant antioxidant defenses, is a crucial process for protecting against oxidative stress known to be associated with a number of pathologies related to burden of life style diseases [1]. This balance is a determinant of physiological processes that ensure the maintenance of healthy cellular function in multiple organs, including the cardiovascular system [2]. Increasing evidence indicates that Nuclear factor erythroid 2-related factor 2 (Nrf2) acts as a key player in this process and that modulation of its action could facilitate both control of cellular redox homeostasis and physiological homeostasis in pathways related to the maintenance of cardiovascular health [3, 4].

Nrf2 is encoded by the nuclear factor erythroid-derived 2-like 2 (NFE2L2) gene, which is a basic-leucine-zipper (bZIP) like transcription factor consisting of seven NRF2-ECH homology (Neh) domains (Neh1-Neh7), belonging to the cap'n'collar (Cnc) subfamily. Its spectrum of action is very broad, regulating the expression of >250 genes [5]. Although Nrf2 is a stress-responsive transcription factor with anti-inflammatory and neuroprotective effects, its major function is to maintain cellular homeostasis by activating genes that encode cytoprotective, antioxidant and phase II detoxifying enzymes, such as NAD(P)H dehydrogenase (quinone)1 (NQO1), heme oxygenase (HO-1) and (HO-2), tryptophan hydroxylase-1 (TPH-1) and glutathione-S-transferase (GST) [6, 7].

Nrf2 is expressed ubiquitously and localized to the cytoplasm under basal conditions. Its expression is maintained at low levels through repression by Kelch-like ECH associated protein1 (Keap1) that functions like a molecular dimmer switch. The interaction between Keap1 and Nrf2 is mediated through the Neh2 domain [8]. Nrf2 activity can be induced by cellular stress, triggering nuclear translocation of Nrf2 and binding to antioxidant response elements (AREs) to orchestrate the transcription of target genes associated with a number of cellular functions including protein homeostasis, redox regulation, iron metabolism, DNA repair and prevention of apoptosis [9, 10].

Nrf2 activity can also be regulated via Keap1 independent, or other pathways **(Fig 1)** [11]. Under basal conditions, Keap1 homodimerizes, and together with the ubiquitination-ligase Cullin-3 (Cul3), inhibits the transcriptional activity of Nrf2 via ubiquitination and proteasomal degradation [12, 13]. Cysteine-rich elements in the protein structure of Keap1 account for its stress sensing activities. In particular, the cysteine C151, C273 and C288 are involved in post-translational modifications, such as oxidation or conjugation to

electrophiles [14]. These cysteine-modifications occurring at its cysteine-thiolate bridge, alleviate the interaction with the Cul3 ligase and therefore diminish Nrf2 proteasomal degradation. Oxidative stress, or Nrf2 activators, enable translocation of Nrf2 from the cytoplasm into the nucleus, where it heterodimerizes with small Maf proteins and transactivates an ARE battery of genes [15].

Recent evidence supports a Keap1 independent mechanism of Nrf2 regulation, whereby the Neh6 domain of Nrf2 plays a crucial role through binding via DSGIS and DSAPGS motifs to β-transducin repeat-containing protein [16]. Another line of evidence suggests a non-canonical pathway for p62-dependent Nrf2 activation, where p62 sequesters Keap1, leading to transactivation of Nrf2-dependent genes [17]. Glycogen synthase kinase 3β (GSK-3β) has also been indicated to modulate the Nrf2 mediated oxidative stress response by promoting Keap1 independent degradation of Nrf2 [18]. In addition, recent evidence show that Nrf2 signaling links endoplasmic reticulum oxidative protein folding and calcium homeostasis in health and disease [19].

Currently, several experimental approaches have evaluated the capacity of Nrf2 to enhance the expression of oxidative stress defense genes and maintain vascular health [3]. However, the role and mode of action of Nrf2 within the complex phenotype of early vascular ageing (EVA) is not well understood. How Nrf2 expression is modulated in response to the structural and functional changes in the cardiovascular system as allostatic load accrues [20], remains to be determined.

For several decades oxidative stress has been recognized as a contributing factor to ageing and ageing-associated pathophysiology [21]. At the cellular level, features of vascular ageing include endothelial cell abnormalities, increased vascular smooth muscle cell (VSMC) growth, vascular inflammation, changes in the quantity and quality of extracellular matrix composition and calcification [22, 23]. Cellular senescence has also been implicated as a hallmark for age related disease [24]. The comprehensive involvement of calcification and senescence in EVA and the role of Nrf2 are discussed below.

1.2. Protective effects of Nrf2

Studies have shown that activation of Nrf2 prior to disease onset maintains general health [25, 26]. Kobayashi *et al.* [27] show that Nrf2 opposes transcriptional upregulation of pro-inflammatory cytokine genes. Calvert *et al.* [28] have demonstrated that hydrogen sulfide (H_2S) mediated Nrf2 expression induces cardioprotective effects, while overexpression of Nrf2 in endothelial cells decreases expression of Interleukin-1 beta (IL-1β), Tumor necrosis factor (TNF), Vascular cell adhesion protein 1 (VCAM1), and Monocyte chemoattractant protein 1 (MCP-1) [29, 30]. Reduced Nrf2 activity leads to higher expression

of pro-inflammatory chemokines and adhesion molecules in endothelial cells [31]. Additionally, activation of Nrf2 has been shown to neutralize oxidative stress in T-lymphocytes, and prevent ischemia-reperfusion (IR) induced acute kidney injury (AKI) [32]. As Nrf2 dampens IR-induced AKI through its protective effects on resident renal epithelial cells [33], this suggests a novel protective mechanism against AKI. Moreover, supplementation of the Nrf2 agonist sulforaphane reduce environmental nephrotoxicity caused by arsenic in rats [34]. Furthermore, in mice with IR injury, activation of the Nrf2 signaling pathway arrested renal interstitial fibrosis [35]. Zheng *et al.* [36] show that natural products isolated from broccoli and cinnamon activate Nrf2 and reduce diabetic renal damage.

Electrophiles derived from polyunsaturated fatty acids, or other organic acids, activate the Nrf2 pathway, predominantly via reversible covalent nucleophile-electrophile modifications on key cysteines in Keap1. Electrophilic nitro-fatty acids inhibit VSMC growth via the activation of the Keap1/Nrf2 pathway [37]. Similar activities have been shown for 15-deoxy-$\Delta_{12,14}$-PGJ2, a pro-resolving prostaglandin metabolite [38] and the lipid electrophile 4-hydroxynonenal (4HNE), which protected against ischemia-reperfusion injury in rodents and cell culture via Nrf2 activation [39, 40]. Another study show that overexpression of thrombomodulin domain-1 in diabetic mice improved renal function via enhancing the Nrf2 antioxidant pathway, resulting in decreased oxidative stress [41]. Additionally, anti-inflammatory effects can ameliorate diabetic nephropathy (DN) in db/db mice through downregulation of the NF-κB mediated pathway [42]. The liver protects itself from harmful chemicals and their potentially damaging metabolites through several defense mechanisms, including the Nrf2/ARE pathway. Moreover, Nrf2 has a protective influence on survival rates and lung integrity in mice [43-45]. Taken together, as the balance between oxidants and antioxidants are crucial for maintaining normal cell signaling and function, Nrf2 has emerged as a major modulator of oxidant stress and implicated in a “Nrf2 diseasome” of chronic burden of life style diseases that are characteristically associated with oxidative stress and inflammation [46, 47].

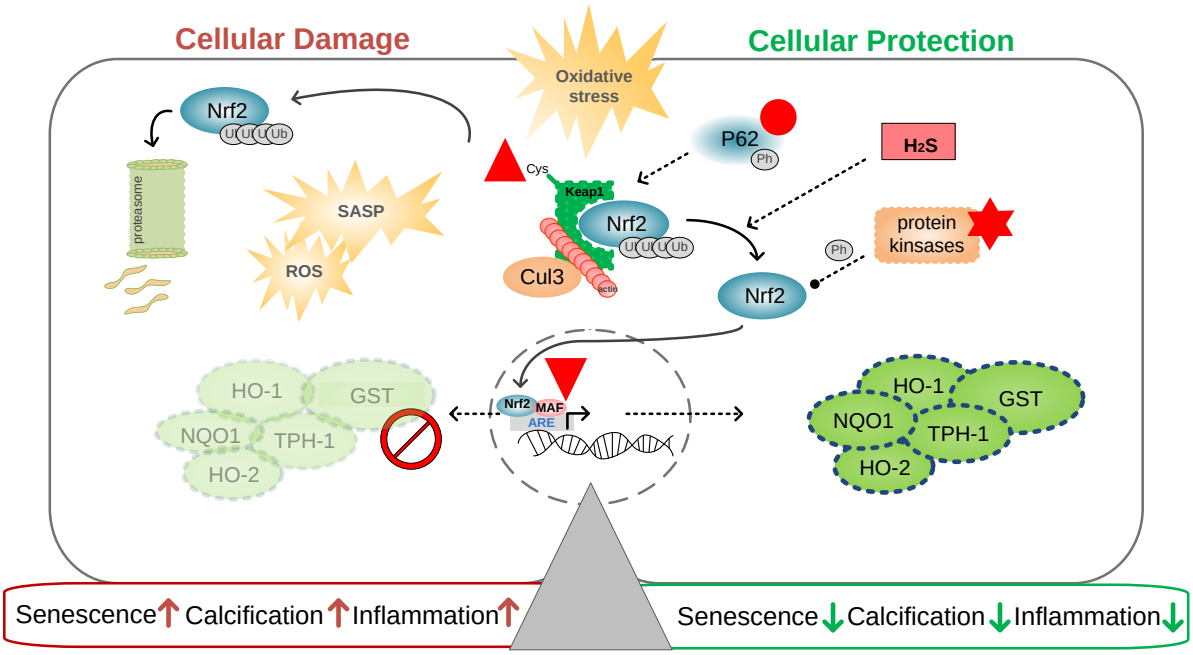

Figure-1: Overview of the role of Nrf2 to regulate the cellular senescence and calcification. Balance of downstream genes of Nrf2 is crucial for the maintenance of cellular homeostasis. Under normal physiological conditions, the cell senses oxidative and inflammatory stress and releases Nrf2 which protects the cell from calcification and cellular senescence via the activation of protective signaling pathways including NQO1, HO-1, HO-2, TPH-1, GST and miRNAs. On the contrary, if the Nrf2 pathway is dysregulated imbalance of Nrf2 response genes occurs and the cell is no longer protected from pro-inflammatory and oxidative stress promoting the development of vascular pathologies. The Nrf2 pathway can be modulated in several steps of the signaling cascade (▲ cysteine modification in Keap1, • p62 activation, ▼ ARE activation, *GSK inhibition). **Abbreviations:** Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, kelch-like ECH associated protein1; ROS, reactive oxygen species; H_2S, hydrogen sulfide; NQO1, NAD(P)H dehydrogenase (quinone) 1; HO-1, heme oxygenase-1; HO-2, heme oxygenase-2; TPH-1, tryptophan hydroxylase-1; GST, glutathione-S-transferase; GSK-3, glycogen synthase kinase 3; SASP, senescence associated secretory phenotype; Cul-3, cullin 3; Ub, ubiquitination; Ph-phosphorylation.

2. Nrf2 in pathological conditions

Insufficient Nrf2-dependent gene expression is associated with a number of distinct pathologies associated with ageing. The development of genetically engineered mouse models of human disease has improved our understanding about the importance of Nrf2 signaling in health and disease. Below in Table 1 we present examples of common diseases associated with EVA and status of Nrf2.

Table-1: Nrf2 impacts on various early vascular ageing associated diseases.

Pathological condition	Mechanism	Findings	Refs

Ageing	Process of becoming older	• Nrf2 dysfunction underlying impaired angiogenesis and microvascular rarefaction in aging	[48]
		• Nrf2 levels decrease with age	[49]
		• Exposure to nano particles induce Nrf2 regulated detoxifying enzymes in young but not older mice cerebellum, liver, and lung	[50]
		• Loss of Nrf2 activity in intestinal stem cells accelerates age-related degeneration of the intestinal epithelium in Drosophila	[51]
Renal disease	Progressive loss of renal function	• Nrf2 knockout mice showed a greater sensitivity to renal damage compared to wild-type mice	[52]
		• Uremic toxin Indoxyl sulfate decrease Nrf2 transcriptional activity in rats	[53]
		• Nrf2 deficiency associated with hypertensive kidney	[52]
		• Impaired Nrf2 activation leads to progression of renal fibrosis	[54]
		• Nrf2 hyperactivation in Keap1 deficient mice showed a bilateral hydronephrosis as indicated by severe bladder swelling	[55]
Atherosclerosis	Narrowing the artery lumen due to build up plaque	• Nrf2 signaling pathway is related with atherosclerosis development	[56]
		• Nrf2 exhibits both pro- and anti-atherogenic effects in experimental animal models	[56]
Hypertension	Elevated blood pressure	• Selective Nrf2 gene deletion in the rostral ventrolateral medulla (RVLM) evokes hypertension and sympathico-excitation in mice	[57]
		• Impaired Nrf2 regulation of mitochondrial biogenesis in RVLM on hypertension induced by systemic inflammation	[58]

Diabetic Cardio-myopathy	Disorder of the heart muscle in diabetes	• Reduced Nrf2 expression observed in the left ventricle of diabetic patient • Nrf2 and its downstream target genes are downregulated in cardiomyocytes from diabetic (db/db) mice	[59] [60]
Cancers	Uncontrolled cell proliferation	• Nrf2 has both tumors suppressive and tumor-promoting effects in cancers	[61]
Renal cancer	Uncontrolled renal cell proliferation	• Modifications of the Nrf2, Keap1-Cul3 complex allow the activation of Nrf2 to aid the survival of tumor • In renal cell cancer, mutations in fumarate hydratase results uncontrolled upregulations of Nrf2 target genes via Keap1	[62] [63] [64]

3. Nrf2 in early vascular ageing

Vascular ageing develops as a progressive modification of vascular function and structure towards increased arterial stiffening. Early vascular ageing can be described as accelerated or aberrant ageing [65], and is a process associated with impairment of physiological functions and an increased risk of further morbidity and mortality [66]. If undetected, EVA leads to vascular stiffening and earlier development of cardiovascular disease (CVD) [65]. The free radical theory of ageing, developed by Harman in the 1950s [67], states that excessive oxidative stress results in ageing through an accumulation of cellular damage. However, since overexpression of antioxidant enzymes, including zinc superoxide dismutase and catalase, does not extend life span in mice [68], oxidative stress might not be a unique mechanism for triggering the vascular ageing process. Increased ROS production induces macromolecular oxidative modifications that promote oxidative damage. With increasing age, oxidative stress accrues, both in humans and animals [69-71] and is closely associated with vascular aging resulting from a failure to activate ARE-driven gene expression and dysregulation of the Nrf2-ARE pathway [72]. In EVA, increased production of ROS promotes endothelial dysfunction, a pathological phenotype associated with the development of stroke, hypertension, atherosclerosis, myocardial infarction and vascular dementia [73].

As a partner in crime, chronic, low grade inflammation ("inflammaging") is strongly linked with oxidative stress [74]. Most age-related 'burden of life style' diseases share an underlying inflammatory component [47, 74]. Although inflammaging correlates with reduced health span [66], its etiology remains to be fully determined. Reducing systemic inflammation and associated oxidative stress has been suggested as a means of mitigating the progression of premature ageing processes [75]. Chronic kidney disease (CKD) is a common condition with underlying premature ageing, due to a complex of toxic alterations in the internal milieu [66]. Kooman *et al.* [75] have proposed four major mechanisms underlying premature ageing in CKD; increase in allostatic load, activation of the stress resistance response, activation of age-promoting mechanisms and impairment of anti-ageing pathways. Patients with CKD are especially vulnerable to EVA associated with cellular senescence, vascular calcification (VC) and depressed Nrf2 expression [76].

3.1. Nrf2 and vascular calcification

Vascular calcification is a pathophysiological process characterized by the deposition of calcium-phosphate crystals in the arteries, typically developing in the intima and media of the vascular wall. The presence of vascular calcification is often detected in CKD, diabetes, atherosclerosis, heart failure and other disorders characterized by changes in vascular structure (i.e. stiffening). The development of calcification is considered to be an active response to the environmental stimuli, such as oxidative stress, inflammation, and changes in passive elements of vascular wall, together with increased phosphate and calcium levels [77]. The role of Nrf2 in the calcification process has been increasingly appreciated due to its regulatory function in the antioxidant and anti-inflammatory pathways [27].

The H_2S donor, sodium hydrosulfide (NaHS) ameliorates calciprotein particles-induced calcification *in vitro* via Keap1/Nrf2 activation system [78]. This inhibitory effect on calcification was achieved by increased expression of the downstream NQO1 gene, and the calcification inhibiting effect was lost and NQO1 expression was reduced when Nrf2 was silenced (**Fig 1**) [78]. Whereas silencing of Keap1 alone does not have a strong impact on calcification, NaHS treatment mediated a significant decrease of TNF mRNA in VSMCs, suggesting anti-inflammatory effects for NaHS [78]. An upregulation of Nrf2 and subsequent increase of HO-1 and -2 expressions was reported in rat aorta upon H_2S treatment [79]. Cell culture experiments show that overexpression of Nrf2 attenuates the process of cellular bone differentiation by interfering with runt-related transcription factor 2 (Runx2) [80]. Thus, Nrf2 deletion results in an increased expression of Runx2 [80]. Since *in vitro* assays using VSMCs treated with the Nrf2 agonist resveratrol show a significantly reduced mineralized matrix deposition, the protection against oxidative stress-induced mitochondrial damage and reduced intracellular calcium deposition could be achieved via Nrf2 and Sirtuin1 signaling [81]. As resveratrol increases the mRNA levels of klotho and Nrf2 in VSMCs

after calcification, this drug may improve the anti-oxidative effect of Nrf2 against hyperphosphatemia-induced calcification [81]. Considering that hyperphosphatemia reduces both mRNA and protein expression of Nrf2 in VSMCs culture [81], high phosphate levels may impair the anti-oxidative role of Nrf2. Indeed, elevated phosphate (even within the normal range) has been associated with poorer outcome [82] with coronary atherosclerosis in young healthy adults [83] and microvascular dysfunction [84]. As tertbutylhydroquinone alleviates high phosphate-induced calcification in VSMCs by suppressing ROS production [85] it is evident that the salutary effects of Nrf2 agonists on VC is not restricted to H_2S and resveratrol and may be a class-effect. Indeed, the classic Nrf2 activator, dimethyl fumarate (DMF) significantly attenuated VC in an *in vitro* ring culture system using mouse thoracic aorta and rat carotid artery [86] under hypercalcemic and hyperphosphatemic conditions. DMF inhibited VC by activating Nrf2 and downregulating osteogenic marker expression in VSMCs [86]. Since Yao *et al.* [87] demonstrated that the Nrf2-ARE signaling pathway enhance the autophagy of VSMC to reduce hyperphosphatemia-induced VC, several mechanisms contribute to the beneficial effects of Nrf2 agonists. It has been proposed that hydrogen peroxide (H_2O_2) can efficiently guard VSMCs against oxidative stress by preventing development of VC triggered by ROS production through Nrf2-ARE pathway [88]. More studies are warranted to understand the complexity of how Nrf2 contribution could be linked to VC and if pharmacological and/or nutraceutical activation of Nrf2 could have beneficial therapeutic effects in groups with high risk of EVA.

3.2. Nrf2 and Senescence

Senescence is characterized as a state of cellular growth arrest, in which the cells are resistant to apoptosis. In essence, senescence acts as an anti-oncogenic mechanism [89]. Although senescent cells are metabolically active they are not positively physiologically contributory to the tissue or organ in which they reside [90]. External stimuli like ROS, high glucose, fatty acid, DNA damage, oncogenes, inflammation and proteotoxic environment act as triggers to render healthy cells senescent [89]. Senescent cells secret a pro-inflammatory senescence associated secretory phenotype (SASP), which is enriched in pro-inflammatory, pro-fibrotic and matrix degrading factors, consisting of chemokines, cytokines, proteases and growth factors that poison the surrounding tissue and contribute to organ dysfunction [91]. One noticeable feature of the SASP is its capacity to activate senescence in neighboring cells via a bystander effect [89]. We have reported that VC in uremic arteries is characterized by increased *CDKN2A/p16INK4*^{a} expression indicating senescence [92].

Numerous studies have investigated the mechanisms behind the age-associated decline in Nrf2 expression in number of different cell types, including bronchial epithelial cells [93], vascular cells [94] and cardiomyocytes [95]. Kuosmanen *et al.* [96] demonstrated that miRNAs derived from senescent cells (most

notably miR-126, miR-21 and miR-100) modulate Nrf2 expression in aged endothelial cells; a process mediated by directly targeting on Nrf2 mRNA **(Fig 1).** It is likely just one of many mechanisms that drive Nrf2 depletion. Moreover, Nrf2 deficiency triggers the development of cellular senescence, as aged Nrf2 double knockout (KO) mice present with increased expression of senescence markers p16INK4a (CDKN2A) and p21 (CDKN1A) compared to wild-type aged mice [97]. Additionally, well-established components of the SASP, such as IL-1β and TNF, are elevated in Nrf2 KO mice [97]. Furthermore, Zhou *et al.* [98] have reported that activation of Nrf2 protects VSMCs against angiotensin II-induced senescence, which was achieved via increased expression of downstream antioxidant genes like HO-1 and NQO1 **(Fig 1)**. Although this suggest a bidirectional relationship between Nrf2 expression and senescence [99], further research is needed to fully elucidate how the repression of this adaptive response is induced and regulated during the ageing processes. The effects of Nrf2 deficiency and its relationship with senescence remain to be comprehensively studied in vascular cells [100], in order to asses possible interplay in diseases characterize by EVA.

It is important to stress that the effects of Nrf2 in CVD and other age-related diseases may be cell-specific [100], as exemplified by a discrepancy in gene expression patterns downstream of Nrf2 in different cell types, when comparing young and old *Drosophila* [51]. This is particularly pertinent, given that drivers of the ageing process are subject to antagonistic pleiotropy. In keeping with this consideration, Nrf2 activation in fibroblasts promote re-epithelialization of skin wounds, as well as gene expression profiles associated with tumorigenic activity also stimulated [101]. Taken together, therapeutic targeting of the Nrf2 pathway should be treated with caution, due to potential antagonistic pleiotropic effects.

4. Clinical implications/ therapeutics/interventions

In the multiple disease entities constituting a ‘diseasome of ageing’ [47, 102], with oxidative stress, mitochondrial dysfunction and persistent inflammation as common underlying features, the Nrf2-Keap1 signaling pathway is frequently disrupted. For example, Ruiz *et al*. [103] demonstrated that CKD-associated diminished antioxidant regulation, was largely caused by impaired activation of Nrf2. Natural Nrf2 activators, such as polyphenols, phytosterols and terpenoids (e.g. Baicalein), mitigate the effects of oxidative stress and reduce inflammation in pre-clinical models of kidney disease, while conversely Nrf2 deletion exacerbated disease pathogenesis and led to autoimmune nephritis [104]. Consequently, restoration and modulation of Nrf2 mediated signaling networks may be effective in retarding CKD progression and EVA [86, 87, 99].

Multiple approaches, such as non-calcium phosphate binders, calcium containing phosphate binders [105, 106], statins [107-109] and vitamin D [110] targeting VC in CKD have been used with varied results and are not curative [111]. A novel approach has been the targeting and removal of senescent cells by senolytic compounds. The use of a senolytic combination (Dasatinib and Quercetin) to specifically remove senescent cells, has been shown to increase the lifespan and improve health span for normatively aged mice [112]. The chronic clearance of senescent cells by combined treatment with Dasatinib and Quercetin has also been shown to alleviate vasomotor dysfunction in normatively aged mice and in mice with established atherosclerosis [113]. This strategy reduced markers of osteogenesis in advanced intimal plaques and ultimately reduced intimal plaque calcification [113]. A recent clinical trial has also reported that treatment with a combination of Dasatinib and Quercetin reduced adipose tissue senescent cell burden in diabetic kidney disease [114].

Specific targeting of the SASP has also proven beneficial. Hegner *et al*. [115] reported that the pharmacological blocking of pro-inflammatory cytokines reduces uremia-induced calcification in vascular progenitor cells *in vitro*. However, several safety and efficacy issues around the removal of senescent cell needs to be addressed. These include the potential for an acceleration of stem cell exhaustion, a hallmark of ageing. In keeping with this, Jeon *et al.* [116] have demonstrated that senescent cells reappeared after the cessation of senolytic treatment in a model of osteoarthritis. This is intuitive, as senescent cells are expected to be generated continuously over the life course in response to exposome stress. Additionally, there is also the possibility that removal of senescent cells without targeting the causes of their accumulation might limit the longer-term benefits of senolytics. The clinical population in which senolytics are targeted also needs to be considered, as it will necessarily include aged and or infirm patients with limited physiological reserve. Without clearance of apoptotic bodies, secondary necrosis could result in the release of pro-inflammatory, signals, further exacerbating the underlying chronic inflammation that occurs in such a population [24]. Another consideration is when in the life-course senolytics can be used. Under the aegis of antagonistic pleiotropy, removal of senescent cells may be appropriate in the later stages of the life-course, but not in the early stages where their requirement for wound healing processes may be desirable [112, 117].

Exploring alternative approaches to target the detrimental effects of senescence without resorting to senolytics therefore need to continue. One such approach would be targeting the SASP. Candidates to suppress or modulate the SASP include rapamycin, NF-κB, or p38 inhibitors [118-121]. Side effects of this approach could include blunting the senescent response, immunosuppression or exacerbation of the accumulation of senescent cells [24].

With the growth in the discovery of Nrf2 activators and regulators, the pharmacological targeting of the regulation of the Nrf2-Keap1 pathway however remains one of the most promising areas of research with multiple drugs or nutraceuticals currently in different stages of clinical trials, (table 2) most notably DMF, bardoxolone-methyl, sulforaphane and curcumin.

Table-2: Nrf2 Modulators in clinical development

Compound	Class	Modulatory effect	Clinical Trial	Clinical Trials.gov identifier
Dimethyl fumarate	Fumaric acid ester	Activator	I, II, III	NCT02784834 NCT02546440 NCT00810836
Bardoxolone-methyl	Synthetic triterpenoids	Activator	I, II, III	NCT00550849 NCT00811889 NCT01351675
Oltipraz	Organosulfur compound	Activator	I, III	NCT00006457 NCT02068339
Ursodio	Biliary acid	Activator	I, II, III, IV	NCT02033876 NCT00200343 NCT01510860
Sulforaphane	Isothiocyanate	Activator	I, II, III, IV	NCT01008826 NCT02880462 NCT02801448 NCT03220542
Curcumin	Stilbene	Activator	I, II, III, IV	NCT02104752 NCT01225294 NCT01052025
Resveratrol	(E)-Stilbene derivative	Activator	I, II, III, IV	NCT01677611 NCT01504854 NCT00743743 NCT02475564

Sulforadex	Sulforaphane/alpha cyclodextrin complex	Activator	I, II	NCT01228084
Ebselen	-	Activator	I, II	NCT03013400
Complexa/ CXA-10	-	Activator	I, II	NCT02248051 NCT03449524 NCT03422510

4.1. Nrf2 pharmacological agonists and inhibitors, the "drugome"

The activation of Nrf2 has been demonstrated to be effective in inhibiting VC in animal models [86, 87, 99]. Various Nrf2 agonists (e.g. Bardoxolone–methyl), have anti-atherogenic and reno-protective effects [122-125], via the upregulation of Nrf2 responsive genes. The main mechanism regulating Nrf2 activity is the control of protein stabilization by Keap1 and most known inducers are electrophilic molecules that covalently modify Keap1 cysteine residues [126].

A range of clinical regulators are in development or trailing [127, 128]. These include DMF which is currently in Phase III trials for the treatment of Multiple sclerosis (MS)s [129]. Activation of Nrf2 by DMF in the central nervous system has been demonstrated in a mouse model of MS. These effects were not observed in Nrf2 null mice, suggesting DMF acts exclusively via the Nrf2 pathway [130]. Bardoxolone methyl, a potent Nrf2 activator and NF-κB suppressor has shown promising results in clinical trials [131], providing enhanced kidney function and delayed onset of ESRD in patients with type-2 diabetes and stage 4 CKD.

Nrf2 has not been considered just a target for treatment, but also for prevention of diseases like cancer thus the role of Nrf2 inhibitors are equally important. In laboratory trials many promising Nrf2 inhibitors have been tested. Different compounds of natural origin have been described to inhabit Nrf2 activity. Alkaloid trigonelline which can be retrieved from coffee beans, is one of these, which has been demonstrated to reduce Nrf2 accumulation into nucleus and ultimately inhibits Nrf2-driven genes transcription [132]. Evergreen shrub *Brucea javanica* extract brusatol is an agent that boosts ubiquitination of Nrf2 and accordingly reduces cytoplasmic Nrf2 levels [133]. Another natural compound mycotoxin ochratoxin A can prevent Nrf2 translocation [134]. Compared with Nrf2 activators data on Nrf2 inhibitors are still preliminary, more basic research and clinical trials are needed to estimate the definite outcome of Nrf2 inhibition.

4.1.1. KEAP1 independent drugs to target Nrf2

Whilst the vast majority of current drugs have typically focused on the interaction of Nrf2 with Keap1, Nrf2 is regulated on multiple levels: transcriptional, epigenetic, covalent protein modification and by proteasome degradation [135]. Several proteins can regulate the Nrf2-ARE pathway, mainly by phosphorylation, and Nrf2 comprises several sites for phosphorylation [136]. Attucks *et al*. [137] have demonstrated *in vitro* that modulation of the transcriptional repressor broad complex-tramtrack-bric a brac and Cap'n'collar homology (BACH) inhibited binding to some ARE-driven genes, independently of Keap1. A failure of redox homeostasis is a hallmark of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD). Several drugs alter the Keap1 independent regulation of Nrf2 in both PD and AD and have been employed in clinical trials with various levels of success [35, 138]. Chen *et al.* subsequently demonstrated that the overexpression of tryptophan hydroxylase-1 (TPH-1), an enzyme involved in metabolite 5-methoxytryptophan, (5-MTP), synthesis, reduced renal injury by attenuating renal inflammation and fibrosis, in a mouse model of CKD via the augmentation of Nrf2 independently of Keap1 [35].

4.1.2. Repurposing of drugs

The use of established therapeutics for new indications has been an area gaining recent attention, especially in the context of cancer treatment [139]. DMF, previously used as a MS and psoriasis drug has demonstrated improvement of MS symptoms in a murine model, via activation of Nrf2 [86, 130, 140]. Metformin, sulfuraphane, both involved in glucose metabolism, statins and GSK-3 inhibitors, have all been earmarked for the treatment of pathologies linked to Nrf2 [141]. Lithium treatment in adulthood or later in life, has been shown to extend lifespan in *Drosophila* via inhibition of GSK-3 and activation of Nrf2 independently of Keap1[142]. Recently Fujiki *et al*. have reported that Tolvaptan, a vasopressin type 2 receptor antagonist can regulate Nrf2 activity via the activation of the Nrf2/HO-1 antioxidant pathway, through phosphorylation of protein kinase RNA-like endoplasmic reticulum kinase [143].

4.2. Challenges and considerations of potential Nrf2 therapeutics

4.2.1. The role of Nrf2 in tumorigenesis

While diminished Nrf2 activity is a hallmark of the diseasome of ageing, its expression is elevated in tumors. As such, the biology of Nrf2 in cancer is complex and context dependent, with Nrf2 demonstrating both anti and pro-tumourgenic properties. It remains to be established if its activity also exhibits hormesis. In non-malignant cells, Nrf2 activation enhances cellular defenses, increasing resistance to oxidant-induced

genetic damage and chemical and physical carcinogens. The activation of a Nrf2 response results in the maintenance of ROS levels below those for signaling proteins critical for tumorigenesis e.g. PI3K, MAPKs and NF-κB. In the early stages of development, due to their enhanced ability to adapt to hostile microenvironment, or high ROS levels, malignant cells with constitutively active Nrf2 in conjunction with various oncogenic pathways, are positively selected. Somatic loss of function mutations in Keap1 or gain of function mutations in NFE2L2 are also common in several tumor types, promoting Nrf2 stability in cancer cells and resulting in unrestrained and sustained Nrf2 activation, [139, 144, 145]. The activation and augmentation of activity of Nrf2 by therapeutics (both synthetic or naturally occurring compounds), is often incongruous with this state of affairs, as such activity is both pulsatile and temporary. Some Nrf2 agonists may also have additional targets with anti-tumorigenic effects [146]. It is, however, encouraging that in the phase III trial of DMF, no difference in cancer rates between placebo and treatment groups was detected [129]. However, it is apparent that any Nrf2 therapeutic treatments will need a safe therapeutic window and require careful monitoring in order to assess potential cancer risk [139, 147].

4.2.2. Animal models, comparative biology and progeroid syndromes

Nrf2 may potentially cause or exacerbate age-related pathology [148]. Modeling the dynamics of Nrf2 in normative ageing is challenging, though Nrf2 null mice share remarkable similarities to old animals [149], including elevated levels of cellular senescence and features of inflammaging. Much insight into the complexity and regulation of the Nrf2 signaling network, can also be gained by research into the structure and function of Nrf2 across species, [47, 102, 150].

4.3. Non-pharmacological Nrf2 therapeutics

4.3.1. Nutrition and Nrf2

Hormesis describes an adaptive, non-monotonic biphasic dose response following an initial disruption in homeostasis. Inflammaging, can be regarded as hormetic stress. Evidence suggests that vitamins, minerals and phytochemicals can act in a hormetic manner [151-153]. Good nutrition can therefore be regarded as a powerful tool to redress the imbalance in pro and anti-inflammatory mediators via the modulation of the Nrf2 network (table 3). Martucci *et al.* [151] have demonstrated this in a project featuring a Mediterranean diet rich in poly unsaturated fatty acids and vegetables rich in nitrate and nitrite, which contribute to endogenous nitro-fatty acid formation and containing polyphenolic Nrf2 activators. Participants showed decreased inflammatory markers and an improved lipid profile via Nrf2 regulation of vitagene and heat shock proteins (Hsp) proteins [151, 154]. These data support growing evidence for the impact of nutrition

on Nrf2-Keap1 signaling pathway [151, 155-157]. Since high salt-loading down-regulates Nrf2 expression in kidney collecting duct cells [158] this suggest that high salt diets should be avoided.

Numerous naturally occurring chemicals derived from plants have anti-inflammatory and antioxidant properties regulated via Nrf2 (table 3). Though generally regarded as weaker Nrf2 agonists compared to synthetic chemicals, several natural compounds have been shown to be potent activators and can induce significant clinical effects [47, 159, 160]. Urolithin A, a metabolite derived from polyphenolics, has been demonstrated to upregulate tight junctions and reduced colitis via Nrf2 agonism [161]. Curcumin has also been shown to be effective in various chronic ageing diseases [162]. Sulforaphane has similarly been shown to have a reno-protective role via Nrf2 activation in diabetic rats subject to oxidative damage, and in Type-2 diabetes patients where it reduced fasting blood glucose levels [36, 151, 163].

Table-3: Nrf2 agonists in naturally occurring compounds

Compound	Class	Mechanism	Food
Sulforaphane	Isothiocyanate	Keap1 cysteine modification stabilize Nrf2	Broccoli Brussel Sprouts Cabbage Cauliflower
Curcumin	Diferuloylmethane	Keap1 thiol modification increase in expression of HO-1	Turmeric
Epigallocatechin gallate	-	Kinase phosphorylation upstream of Nrf2 increased HO-1 expression	Green tea
Allyl sulfides	Organosulfur compounds	Keap1 cysteine modification	Garlic
Resveratrol	Polyphenol: allyl sulfides	Kinase phosphorylation upstream of Nrf2	Grapes Red wine
Lycopene	Phytochemical: tetraterpene carotenoid	Increase Nrf2/HO-1 expression	Tomatoes Carrots
Caspaicin	Phytochemical	Inhibition of NQO1	Chilies
Fisetin	Flavonoid	Multiple e.g. increased glutothione expression neutralisation of reactive oxygen species disruption of the PI3K/AKT pathway	Strawberries Apples Onions Cucumber

Quercitin	Flavonoid	Increased HO-1 and NQO1 expression	Red kidney bean Caper Radish onion
Cinnamaldehyde	Unsaturated aldehyde	Increased Nrf2 expression Increased Nrf2 nuclear translocation Suppressed NF-κB activation	Cinnamon

4.3.2. The gut microbiome and Nrf2 activation

CKD is characterized by an altered gut microbiome, resulting in an accumulation of uremic toxins such P-cresyl sulfate (PCS) and indoxyl sulfate (IS) [157, 164]. PCS and IS are uremic toxins that are directly related to accelerated progression of CKD, and both are derived from the colonic bacterial fermentation of dietary protein, [160]. Mafra *et al.* [165] have demonstrated that the dysbiosis in the gut microbiome in CKD leads to an increase in the bacteria that generate the uremic toxins IS, PCS, Indole-3-acetic acid, (IAA), and Trimethylamine (TMA), leading to inflammation. Uremic toxins affect gene expression via NF-κB and Nrf2 regulated pathways [53]. Lau *et al.* [166] have demonstrated that uremic toxins from gut microbiota accentuate the Nrf2/NF-κB imbalance and have proposed the use of probiotics, prebiotics and symbiotics to reduce toxin levels in CKD patients, and hence their risk of EVA.

Urolithin A, is a major microbial metabolite which displays anti-inflammatory, anti-oxidative, and anti-cellular ageing activities, that has been shown to upregulate gut barrier epithelial tight junction proteins via Nrf2 mediated activity [161]. Alteration of the composition of gut microbiota via diet/supplements could therefore be an effective non-pharmacological means of modulating the level of oxidative stress via Nrf2 activation. This has been demonstrated by the restriction of protein in the diet of CKD patients. A low protein diet reduce levels of oxidative stress and inflammation via the modulation of Nrf2 expression in non-dialysis CKD patients [156, 160, 167], and has been hypothesized to modulate the gut microbiota and reduce the generation of uremic toxins, such as PCS and IS [160]. The source of protein within the diet has also been shown to be significant, with plant protein intake being associated with lower production of uremic toxins and lower serum phosphorus and warrants further exploration, [168, 169].

4.3.3. Exercise

Exercise is an effective modulator of Nrf2. Exercise induces ROS, increasing the level of oxidative stress, which results in an increased dissociation of Nrf2 from Keap1 [170]. Several groups have demonstrated an

increase in Nrf2 expression in old rats following exercise [171, 172]. Subsequently Abreu *et al*. [173] have demonstrated Nrf2 induction and inhibition of NF-κB following resistance exercise in CKD patients undergoing haemodialysis. The Nrf2 response to exercise however varies according to training modality, duration and age [95]. Although acute exercise increased Nrf2 protein levels in peripheral blood mononuclear cells in young and older men, nuclear accumulation of Nrf2 was observed only in the young group. This indicates that ageing *per se* is accompanied by a reduced nuclear import of Nrf2 [174, 175]. Exercise has also been shown to induce epigenetic changes, predominantly altering the methylation pattern of the promoters of genes in the adaptive antioxidant response, [176]. Overall, exercise is a realistic intervention to improve endogenous antioxidant defenses via Nrf2 activation, though this must be viewed in the context of antagonistic pleiotropy to yield maximum benefit.

5. Conclusions

Premature vascular aging is a common problem for both general populations and patients with chronic inflammatory diseases like CKD or atherosclerosis. Two major factors that drive healthy cells towards early vascular aging are calcification and senescence, both arising from oxidative stress and persistent low-grade inflammation. These conditions are characterized by a failure to activate ARE-driven gene expression and a dysregulation of the Nrf2-ARE pathway. As reviewed here, a multitude of studies suggest that modulating Nrf2 activation is beneficial for targeting the burden of lifestyle diseases. Nrf2 activation might be induced by pharmacological treatments or non-pharmacological therapeutics such as diet and exercise. Targeting Keap1 pharmacologically, thereby controlling Nrf2 protein stabilization, is proposed as the most auspicious intervention. Several clinical regulators currently in clinical development or trialing for example DMF and Bardoxolone methyl, have shown promising results. Besides the direct interaction with Keap1, Nrf2 is regulated on multiple levels, including transcriptional, epigenetic, covalent protein modification and proteasome degradation. A few Keap1 independent targets have been described to modulate the Nrf2-ARE pathway, including the transcriptional repressor BACH, or TPH-1. Despite the promising results in preclinical research and clinical trials, safety concerns regarding overexpression have surfaced in recent years, regarding the role of Nrf2 in cancer development and potential side effects of Nrf2 activation in a complex tumor environment. These concerns require additional address. Nevertheless, Nrf2 constitutes a powerful target for intervention in premature ageing, whether as sole treatment or as adjuvant treatment in combination with selective therapies targeting of senescence or calcification.

Figure legends

Figure-1: Overview of the role of Nrf2 to regulate the cellular senescence and calcification. Balance of downstream genes of Nrf2 is crucial for the maintenance of cellular homeostasis. Under normal physiological conditions, the cell senses oxidative and inflammatory stress and releases Nrf2 which protects the cell from calcification and cellular senescence via the activation of protective signaling pathways including NQO1, HO-1, HO-2, TPH-1, GST and miRNAs. On the contrary, if the Nrf2 pathway is dysregulated imbalance of Nrf2 response genes occurs and the cell is no longer protected from pro-inflammatory and oxidative stress promoting the development of vascular pathologies. The Nrf2 pathway can be modulated in several steps of the signaling cascade (▲ cysteine modification in Keap1, • p62 activation, ▼ ARE activation, *GSK inhibition). **Abbreviations:** Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, kelch-like ECH associated protein1; ROS, reactive oxygen species; H_2S, hydrogen sulfide; NQO1, NAD(P)H dehydrogenase (quinone) 1; HO-1, heme oxygenase-1; HO-2, heme oxygenase-2; TPH-1, tryptophan hydroxylase-1; GST, glutathione-S-transferase; GSK-3, glycogen synthase kinase 3; SASP, senescence associated secretory phenotype; Cul-3, cullin 3; Ub, ubiquitination; Ph-phosphorylation.

Table-1: Nrf2 impacts on various early vascular ageing associated diseases.

Table-2: Nrf2 Modulators in clinical development**.**

Table-3: Nrf2 agonists in naturally occurring compounds.

Author Contributions: S.A, S.B., S.H, J.S. and S.A. searched the literature and drafted the review. K.K., P.G.S. and P.S. contributed important intellectual content and editing during manuscript drafting and revision.

Acknowledgments: This work was supported by grants from Swedish Research Council grant no. 2018–00932; Strategic Research Program in Diabetes at Karolinska Institutet, Sweden; funding from the European Union (INTRICARE and CaReSyAn); CIMED (centrum för innovativ medicin); Njurfonden, Sweden; Hjärt lungfonden, Sweden. The content of this article reflects only the views of its authors.

Conflicts of Interest: Peter Stenvinkel is in the scientific advisory board of REATA.

Other authors have no conflict of interest to declare.

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