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Mediators in cardiovascular disease: association with Raynaud's phenomenon

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Mediators in cardiovascular disease: Association with Raynaud's phenomenon

Amaal Eman Abdulle

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Mediators in cardiovascular disease: association with Raynaud's phenomenon

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1

CHAPTER

General Introduction

Vascular damage (i.e. vasculopathy), a general term used to describe a broad spectrum of diseases affecting the blood vessels, is often an overlooked component of many diseases. Numerous diseases such as metabolic conditions, inflammation, oxidative stress, and auto-immune disorders alter the function and structure of the vasculature. Although vascular injury may directly arise from different etiologies, addressing risk factors associated with the development of vasculopathy is most important, because it is a common denominator in the pathophysiology of cardiovascular disease. A better understanding of the development of vascular damage in general may promote the development of therapeutic interventions.

Depending on the underlying aetiology, any layer of the vessel wall (i.e., adventitia, media, and/or intima) may be affected. While there are similarities in the pathologic changes of many vascular diseases, there are also many distinct differences. Vasculopathy is generally divided into macro- and microvascular damage. In systemic sclerosis (SSc), primarily small and medium sized arteries are affected, often leading to detrimental outcome (e.g. digital ulcers and necrosis), for which a cure has yet to be found.

Raynaud's phenomenon (RP), a disorder of the microvasculature which causes poor blood flow to the digits, is usually the first recognizable sign of SSc. The aetiology of vascular pathology in SSc is unknown, but there are several ways in which vascular injury could occur in SSc. This includes infectious agents, free radicals, cytotoxic T cells, and auto-antibodies directed against endothelial cells.¹ In addition, patients with SSc may have an increased risk of developing atherosclerosis that is secondary to vascular wall changes. Several studies have shown an increased prevalence of distal peripheral vascular disease in patients with SSc compared to healthy controls.² Therefore, given the fact that RP is an easily recognizable feature of vasculopathy, and may be seen as a mirror for more widespread systemic vasculopathy in SSc, it is of utmost importance to further study this phenomenon in order to gain more insight into this complex disease.

RAYNAUD'S PHENOMENON

Raynaud's phenomenon can be primary (PRP) or secondary (SRP) to several diseases or conditions (e.g., connective tissue disease (CTD), use of medication, and exposure to vibration).³ Although the exact pathogenesis of RP remains unknown, early recognition of SRP could substantially decrease the time to diagnosis of the underlying disease, and thereby possibly attenuate disease course by an early start of treatment. An RP attack is characterized by a distinctive bi/triphasic colour pattern in the affected tissue, causing pallor, cyanosis, and/or rubor. It was previously reported that the prevalence of RP varies between 4-20%.^{4,5} This variation is strongly dependent on the geographical location, with estimates being higher in colder regions.⁶ The clinical severity of RP varies greatly, ranging from a simple but uncomfortable condition to severe symptoms that seriously affect the patient's quality of life.⁷

Raynaud's phenomenon is often the most recognizable sign of SSc. SSc is a systemic disease characterized by inflammation, the formation of specific antibodies, vascular damage, and the formation of excessive fibrosis in the skin and internal organs. The prevalence rate of SSc has been reported to range from 30 to 300 cases per 1 million persons.^{8–11} However, exact numbers of earlier stages of the disease are missing, which may be attributed to the fact that the initially developed internationally used classification criteria of SSc, the ACR/EULAR criteria, have been previously shown to have a low sensitivity for the detection of early SSc.^{12–14}

Although RP occurs in the majority of cases in healthy individuals, a physician may suspect SSc based on other symptoms. In this case, blood tests and other procedures (e.g., nail-fold capillaroscopy) can be performed in order to confirm, or rule out SSc. Early discovery of SSc is thought to allow for an appropriate implementation of an effective therapy regimen to stop disease development. Although more attention should be paid to the early identification of SSc in order to optimally use “the window of opportunity”, the greatest challenge still remains the search for valid predictors of disease initiation and progression.¹⁵

PATHOPHYSIOLOGY OF RAYNAUD'S PHENOMENON

Although the pathogenesis and risk factors of RP are undoubtedly diverse and complex, it should be noted that there are distinct differences in the pathophysiology of PRP and SRP. There is substantial evidence in PRP that abnormal alpha-adrenergic responses, particularly mediated by alpha-2 adrenoreceptors, play a role in the enhanced sensitivity to cold temperatures. Alpha-1 and alpha-2 adrenoreceptors are found on the vascular smooth muscle of human extremity arteries in normal subjects, with alpha-2 adrenoreceptors being more prominent on distal arteries.¹⁶ Local cooling increases vasoconstriction mediated by alpha-2 receptors, especially the alpha-2C subtype.¹⁷ Several other factors are also thought to play an important role in the occurrence of PRP. For instance, the majority of patients with PRP are young pre-menopausal females. This clearly reveals a gender-based component and suggests that sex-hormones may play a role in the onset of this disease. Moreover, it was previously shown that the incidence of RP-like vasospastic reactions increase in the luteal phase during the menstrual cycle.¹⁸ Collectively, these findings indicate that hormonal factors may (partially) account for the higher prevalence of RP in women.¹⁹ In addition to the hormonal factors, genetic factors have also been implicated in the development of RP. It was previously reported that approximately 30–50% of the patients with PRP have a first-degree relative with the condition.²⁰ Moreover, RP was found to be more common among monozygotic twins than dizygotic twins.²¹

In SRP, it is assumed that an underlying vascular disease disrupts the usual mechanisms that regulate vessel reactivity. As endothelial function is impaired, vascular function is compromised, resulting in a substantial reduction in nutrient flow and a risk of essential tissue ischemia. It is commonly believed that cold-induced vasoconstriction in SRP can be the result of several factors including increased sympathetic nerve activity (and amplified sympathetic response), environmental factors (vibration injury and emotional stress), oxidative stress, and genetic factors.^{22,23}

In order to maintain proper redox homeostasis, reactive oxygen species (ROS) serve as crucial signaling molecules that regulate a variety of physiological activities. Oxidative stress is defined as an imbalance between the formation of ROS and the antioxidative response, with ROS predominating.^{24,25} The recurrent vasospastic events, which induce hypoxia followed by reperfusion, can lead to an increased production of ROS.²⁶ In SSc, oxidative stress may cause the activation and damage of endothelial cells (ECs),²⁵ leading to EC apoptosis and impairment of cell-cell adhesion.²⁷ This impairment ultimately increases vascular endothelial permeability, and eventually alters the EC signal transduction.²⁸ When vascular ECs are injured, they produce more vasoconstrictive mediators (e.g., endothelin-1 [ET-1]) and also have a reduced ability to release NO and prostacyclin.^{29–31} This imbalance in mediators, causes an altered vascular tone, which may initiate a self-perpetuating cycle of recurrent RP attacks, increased ROS production, more injured ECs, and an inability to defend against oxidative stress.^{26,32–34}

In addition to the above mentioned factors, some epidemiological studies have demonstrated the relationship between body weight and the occurrence of RP.^{35,36} For instance, the loss of substantial body weight could increase a person's susceptibility to RP. However, current literature has not reached a consensus on the exact role of body weight. For instance, one study demonstrated that underweight women were not at increased risk of RP,³⁵ however, several additional case reports, on the other hand, have already indicated that RP can occur in conjunction with specific eating disorders.^{37,38} Furthermore, previous research has shown that participants with anorexia nervosa have an increased level of oxidative stress.^{39,40} However, the exact relationship between low body weight, oxidative stress and the occurrence of SSc-related vasculopathy (such as RP) has to be further elucidated.

SCOPE OF THE THESIS

The aim of this thesis is two-fold. First, we aim to explore factors associated with the occurrence of RP in the general population and investigate the role of low body weight in the occurrence of RP and nail-fold capillary abnormalities. The second part of this thesis will provide an overview of the role of redox status (as an indirect measure of oxidative stress) as readout of health and disease (i.e., RP).

The first part of this thesis will focus on the epidemiology of RP and the role of low body weight in the occurrence of primary RP and nail-fold capillary abnormalities. Although several studies have previously reported on the epidemiology of RP, exact numbers in the general Dutch population are lacking. In **Chapter 2** we will focus on the epidemiology of RP and its relationship with low body weight and involuntary weight loss. Given the fact that it was previously reported that severely underweight subjects (e.g., anorexia nervosa) might develop vasculopathy-like condition in the absence of an underlying connective tissue disease, it could be hypothesized that factors related to body weight play an important role in the development of nailfold capillaroscopic abnormalities in PRP. Therefore, in **Chapter 3** we will investigate the association of low body weight in

the occurrence of nailfold capillaroscopic abnormalities in subjects with RP, without an underlying CTD. We further hypothesize that the vascular changes in subjects with a low body weight could be due to increased oxidative stress. This postulation is supported by a previous study demonstrating that endothelium-dependent vasodilation is impaired in subjects with a low BMI (compared with normal BMI), due to reduced bioavailability of nitric oxide.⁴¹ Therefore, in the second part of this thesis we will focus on the role of redox status in both RP and healthy conditions. **Chapter 4** comprises a more extensive introduction to the role of oxidative stress in the development of SSc-related vasculopathy. Given the relation between oxidative stress and disease in general, in **Chapter 5** we aimed to establish the predictive value of free thiols (as a measure of oxidative stress) for cardiovascular events and mortality in the general population. In **Chapter 6** we will investigate the response of the redox system to a standardized cooling experiment in subjects with PRP, SSc and healthy controls. In **Chapter 7** we review hydrogen sulfide as a therapeutic option in SSc-related vasculopathy. In **Chapter 8** the content of this thesis is summarized and discussed, followed by our perspective on the future direction of studies focused on the pathogenesis of RP and the role of redox-related approaches in the treatment of RP.

REFERENCES

1. Kaviani N, Batteux F. Macro- and microvascular disease in systemic sclerosis. *Vascul Pharmacol*. 2015;(71):16-23. doi:10.1016/j.vph.2015.05.015
2. Veale DJ, Collidge TA, Belch JFF. Increased prevalence of symptomatic macrovascular disease in systemic sclerosis. *Ann Rheum Dis*. 1995;(54):853-855. doi:10.1136/ard.54.10.853
3. Wigley FM, Flavahan NA. Raynaud's Phenomenon. *N Engl J Med*. 2016;375(6):556-565. doi:DOI: 10.1056/NEJMra1507638
4. Voulgari P V., Alamanos Y, Papazisi D, Christou K, Papanikolaou C, Drosos AA. Prevalence of Raynaud's phenomenon in a healthy Greek population. *Ann Rheum Dis*. 2000;59:206-210. doi:10.1136/ard.59.3.206
5. Çakır N, Pamuk Ö N, Dönmez S, et al. Prevalence of Raynaud's phenomenon in healthy Turkish medical students and hospital personnel. *Rheumatol Int*. 2008;29:185-188. doi:10.1007/s00296-008-0666-9
6. Fraenkel L. Raynaud's Phenomenon: Epidemiology and Risk Factors. *Curr Rheumatol Rep*. 2002;4(2):123-128.
7. Pope JE. The diagnosis and treatment of Raynaud's phenomenon: A practical approach. *Drugs*. 2007;67(14):517-525. doi:10.2165/00003495-200767040-00003
8. Mayes MD, Lacey J V., Beebe-Dimmer J, et al. Prevalence, incidence, survival, and disease characteristics of systemic sclerosis in a large US population. *Arthritis Rheum*. 2003;48(8):2246-2255. doi:10.1002/art.11073
9. Ranque B, Mouthon L. Geoepidemiology of systemic sclerosis. *Autoimmun Rev*. 2010;9(5):A311-A318. doi:10.1016/j.autrev.2009.11.003
10. Vonk MC, Broers B, Heijdra YF, et al. Systemic sclerosis and its pulmonary complications in The Netherlands: An epidemiological study. *Ann Rheum Dis*. 2009;68(6):961-965. doi:10.1136/ard.2008.091710
11. Chiffot H, Fautrel B, Sordet C, Chatelus E, Sibilia J. Incidence and Prevalence of Systemic Sclerosis: A Systematic Literature Review. *Semin Arthritis Rheum*. 2008;37(4):223-235. doi:10.1016/j.semarthrit.2007.05.003
12. Scussell Lonzett L, Joyal F, Raynauld JP, et al. Updating the American College of Rheumatology preliminary classification criteria for systemic sclerosis: Addition of severe nailfold capillaroscopy abnormalities markedly increases the sensitivity for limited scleroderma. *Arthritis Rheum*. 2001;44(3):735-736. doi:10.1002/1529-0131(200103)44:3<550::AID-ANR103>3.0.CO;2-5
13. Leroy EC, Black C, Fleischmajer R, et al. Scleroderma (Systemic Sclerosis): Classification, Subsets and Pathogenesis. *J Rheumatol*. 1988;15(2):202-205.
14. LeRoy EC, Medsger J. Criteria for the classification of early systemic sclerosis. *J Rheumatol*. 2001;28(7):1573-1576.
15. Guiducci S, Bellando-Randone S, Matucci-Cerinic M. A new way of thinking about systemic sclerosis: The opportunity for a very early diagnosis. *Isr Med Assoc J*. 2016;18(3-4):141-143.
16. Flavahan NA, Cooke JP, Shepherd JT, Vanhoutte PM. Human postjunctional alpha-1 and alpha-2 adrenoceptors: Differential distribution in arteries of the limbs. *J Pharmacol Exp Ther*. 1987;241(2):361-365.
17. Sawasaki N, Iwase S, Mano T. Effect of skin sympathetic response to local or systemic cold exposure on thermoregulatory functions in humans. *Auton Neurosci Basic Clin*. 2001;87(2-3):274-281. doi:10.1016/S1566-0702(00)00253-8
18. Bartelink ML, Wollersheim H, Theeuwes A, Van Duren D, Thien T. Changes in skin blood flow during the menstrual cycle: The influence of the menstrual cycle on the peripheral circulation in healthy female volunteers. *Clin Sci*. 1990;78:527-532. doi:10.1042/cs0780527
19. Nussinovitch U, Shoenfeld Y. The role of gender and organ specific autoimmunity. *Autoimmun Rev*. 2012;11(6-7):A377-A385. doi:10.1016/j.autrev.2011.11.001

20. Freedman RR, Mayes MD. Familial aggregation of primary Raynaud's disease. *Arthritis Rheum.* 1996;39(7):1189-1191. doi:10.1002/art.1780390717
21. Cherkas LF, Williams FMK, Carter L, et al. Heritability of Raynaud's phenomenon and vascular responsiveness to cold: A study of adult female twins. *Arthritis Care Res.* 2007;57(3):524-528. doi:10.1002/art.22626
22. Flavahan NA. A vascular mechanistic approach to understanding Raynaud phenomenon. *Nat Rev Rheumatol.* 2015;11(3):146-158. doi:10.1038/nrrheum.2014.195
23. Herrick AL. Pathogenesis of Raynaud's phenomenon. *Revmatologija.* 2005;13(3):62-65. doi:10.1093/rheumatology/keh552
24. Betteridge DJ. What is oxidative stress? *Metabolism.* 2000;49(2 Suppl 1):3-8. doi:10.1016/S0026-0495(00)80077-3
25. Grygiel-Górniak B, Puszczewicz M. Oxidative Damage and Antioxidative Therapy in Systemic Sclerosis. *Mediators Inflamm.* 2014;1-11. doi:10.1155/2014/389582
26. Herrick AL, Matucci Cerinic M. The emerging problem of oxidative stress and the role of antioxidants in systemic sclerosis. *Clin Exp Rheumatol.* 2001;19(1):4-8.
27. Sgonc R, Gruschwitz MS, Dietrich H, Recheis H, Gershwin ME, Wick G. Endothelial cell apoptosis is a primary pathogenetic event underlying skin lesions in avian and human scleroderma. *J Clin Invest.* 1996;98(3):785-792. doi:10.1172/JCI118851
28. Fleischmajer R, Perlish JS. Capillary alterations in scleroderma. *J Am Acad Dermatol.* 1980;2(2):161-170. doi:10.1016/S0190-9622(80)80396-3
29. Freedman RR, Girgis R, Mayes MD. Endothelial and adrenergic dysfunction in Raynaud's phenomenon and scleroderma. *J Rheumatol.* 1999;26(11):2386-2388.
30. Romero LI, Zhang DN, Cooke JP, et al. Differential expression of nitric oxide by dermal microvascular endothelial cells from patients with scleroderma. *Vasc Med.* 2000;5(3):147-158.
31. Matucci Cerinic M, Kahaleh MB. Beauty and the beast. The nitric oxide paradox in systemic sclerosis. *Rheumatology (Oxford).* 2002;41(8):843-847.
32. Servettaz A, Goulvestre C, Kavian N, et al. Selective oxidation of DNA topoisomerase 1 induces systemic sclerosis in the mouse. *J Immunol.* 2009;182(9):5855-5864. doi:10.4049/jimmunol.0803705
33. Chung CP, Avalos I, Stein CM. Oxidative stress, microvascular dysfunction, and scleroderma: An association with potential therapeutic implications, a commentary on "Postocclusive reactive hyperemia inversely correlates with urinary 15-F2t-isoprostane levels in systemic sclerosis." *Free Radic Biol Med.* 2006;40(10):1698-1699. doi:10.1016/j.freeradbiomed.2006.03.001
34. Ogawa F, Shimizu K, Muroi E, et al. Serum levels of 8-isoprostane, a marker of oxidative stress, are elevated in patients with systemic sclerosis. *Rheumatology.* 2006;45(7):815-818. doi:10.1093/rheumatology/keh012
35. Harpsøe MC, Basit S, Andersson M, et al. Body mass index and risk of autoimmune diseases: A study within the Danish National Birth Cohort. *Int J Epidemiol.* 2014;43(3):843-855. doi:10.1093/ije/dyu045
36. Maricq HR, Carpentier PH, Weinrich MC, et al. Geographic variation in the prevalence of Raynaud's phenomenon: a 5 region comparison. *J Rheumatol.* 1997;24(5):879-889.
37. Yucel B, Kubat Uzum A, Ozbey N, Kamali S, Yager J. Anorexia Nervosa and Raynaud's Phenomenon: A Case Report. *Int J Eat Disord.* 2007;40(2):762-765. doi:10.1002/eat
38. Sachs K V, Harnke B, Mehler PS, Krantz MJ. Cardiovascular complications of anorexia nervosa: A systematic review. *Int J Eat Disord.* 2016;49(3):238-248. doi:10.1002/eat.22481
39. Higashi Y, Sasaki S, Nakagawa K, et al. Low body mass index is a risk factor for impaired endothelium-dependent vasodilation in humans: Role of nitric oxide and oxidative stress. *J Am Coll Cardiol.* 2003;42(2):256-263. doi:10.1016/S0735-1097(03)00630-2

2

CHAPTER

Low body weight and involuntary weight loss are associated with Raynaud's phenomenon in both men and women

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ABSTRACT

Objectives: Low body weight is an easily assessable cause of Raynaud's phenomenon (RP), and is frequently overlooked by clinicians. We aim to investigate the association of low body weight (BMI<18.5 kg/m²), involuntary weight loss, and nutritional restrictions with the presence of RP.

Methods: Participants from the Lifelines Cohort completed a validated self-administered connective tissue disease questionnaire. Subjects who reported cold-sensitive fingers and bi- or triphasic colour changes were considered to suffer from RP. Patient characteristics, anthropometric measurements, and nutritional habits were collected. Statistical analyses was stratified for gender.

Results: 93,935 participants completed the questionnaire. Prevalence of RP was 4.2% [95% CI 4.1-4.4], and was three-fold higher in women compared to men (5.7% vs. 2.1%, $p<0.001$). Subjects with RP had a significantly lower daily caloric intake compared to subjects without RP. Multivariate analysis, correcting for creatinine level, daily caloric intake and other known etiological factors associated with RP, revealed that low body weight (men: OR 5.55 [95% CI 2.82-10.93]; women: OR 3.14 [95% CI 2.40-4.10]), and involuntary weight loss (men: OR 1.32 [1.17-1.48], women: OR 1.31 [1.20-1.44]) were significantly associated with the presence of RP. In addition, low-fat diet was associated with RP in women (OR 1.27 [1.15-1.44]).

Conclusion: Low body weight and prior involuntary weight loss are associated with an increased risk of RP in both men and women. The current study emphasizes that low body weight and weight loss are easily overlooked risk factors of RP, and should be assessed and monitored in subjects with RP.

INTRODUCTION

Raynaud's phenomenon (RP), which is a common disorder, is characterized by an episodic discoloration of the extremities in response to cold exposure or emotional stress.^{1,2} Raynaud's phenomenon is common in young females and may be associated with several underlying connective tissue diseases (CTD) including systemic sclerosis. Although patients presenting with RP are usually screened for features of CTDs, easily measurable causes of RP, such as low body weight or recent weight loss, are often overlooked.

In clinical practice, many patients with RP are underweight, recently lost weight, or have dietary restrictions, potentially leading to malnutrition. Several case reports have demonstrated that RP can concomitantly occur in certain eating disorders.^{3,4} In addition, it was reported that patients with anorexia nervosa suffering from RP exhibit nail-fold capillaroscopic findings typical of a connective tissue disease.⁵ Collectively, these findings suggest that low body weight increases a patient's susceptibility to RP. However, studies investigating the relationship between low body weight, weight loss and RP are lacking. The objective of the current study is to investigate the association of low body weight, involuntary weight loss, and dietary restrictions, with the presence of RP in the general population. In addition, we will investigate other factors that are associated with low body weight, including diets based on low-fat and low-carbohydrate intake. In order to get a clear understanding on the relationship between low body weight and RP, we will adjust for factors associated with low body weight (e.g., creatinine level (as a surrogate of muscle mass), daily caloric intake) and other known etiological factors associated with RP (i.e., age, hormonal status (in women), known CTD, smoking, and use of medication).

MATERIAL AND METHODS

PARTICIPANTS

In this cross-sectional study, we analyzed data from the LifeLines Cohort Study. “LifeLines is a prospective multidisciplinary population-based cohort study examining in a unique three-generation design the health and the health-related behaviors of 167,729 persons living in the North of the Netherlands”.⁶ Inclusion of the participants started in 2013, and all participants will be followed for 30 years. Prior to the first visit at the LifeLines outpatient clinic, all participants were asked to complete a self-administered questionnaire, and to provide (self-reported) information about their medical history, current diseases, use of medication, and health behavior. During the first visit all participants underwent a general clinical examination, followed by blood and urine collection. All participants were aged ≥ 18 years, and subjects were only excluded from the current study if the connective tissue disease screening questionnaire (details see below) was missing. The Lifelines Cohort study was conducted according to the principles of the Declaration of Helsinki, approved by the local medical ethics review committee of the University Medical Center Groningen (UMCG), and all participants provided written informed consent to participate in this study.

DATA COLLECTION

The LifeLines Cohort is a comprehensive source of demographic, physical, biomedical, behavioral, and psychological data. Information on data access procedure can be found at <http://www.Lifelines.nl>. For the current study several baseline patient characteristics were obtained, e.g., gender, age, smoking behavior (current smoking was defined as smoking at the time of study entry or in the past month), and hormonal status (i.e., pre vs. post-menopausal, receiving hormonal contraceptives, receiving hormonal treatment other than contraceptives), and medication use (beta-blocking agents, immunosuppressive drugs, and central acting sympathomimetic).

CONNECTIVE TISSUE DISEASE SCREENING QUESTIONNAIRE

LifeLines participants completed a number of questionnaires including CTD screening questionnaire during the follow-up assessment, which had taken place within 3 years following the baseline assessment (median 25 months [IQR 23-31]). This validated questionnaire comprised 30 questions designed at identifying potential CTDs, including the presence of RP.^{7,8} The two panel methodology was applied to translate the English CTD questionnaire. A bilingual panel agreed on the best Dutch translations for the items. Subsequently, the questionnaire was presented to a lay panel for consideration. This panel consisted of both men and women of different ages and average level of education (non-medical) to ensure that the language being used in the final version would be understood by potential respondents, and that it was in everyday Dutch. The presence of RP was assessed using five specific questions of the CTD questionnaire, namely: “Are your fingers unusually sensitive to the cold?” (question 1), “Have your fingers ever shown

any unusual colour changes in the cold?" (question 2), "If your fingers have ever shown any unusual colour changes in the cold, was the colour white?" (question 3), "If your fingers have ever shown any unusual colour changes in the cold, was the colour blue/purple?" (question 4), and "If your fingers have ever shown any unusual colour changes in the cold, was the colour red?" (question 5). Subjects who answered the first two questions (question 1 and question 2) with a "yes" were considered to possibly be suffering from RP, and participants who answered both questions with a "yes" and reported bi- or triphasic discoloration of the fingers were classified as definite RP.⁹ In addition, subjects who answered the first two questions with a "no" were considered as non-RP participants.

DIETARY ASSESSMENT

In order to assess factors associated with low body weight a 110-item food frequency questionnaire was used. This questionnaire was designed to assess food intake over the previous month.¹⁰ In addition, daily energy and macronutrients intake (daily caloric intake (kcal), daily intake of carbohydrates (g/day), and daily intake of fat (g/day)) was calculated based on the answers provided on the food frequency questionnaire at baseline. Low-fat diet was defined as a diet in which <30% of the total calories was derived from fat,^{11,12} low-carbohydrate diet was defined as a diet in which <26% of the total calories was derived from carbohydrates,¹³ and low-calorie intake was defined as <1000 calories per day. Serum creatinine was measured on a Roche Modular P chemistry analyzer (Roche, Basel Switzerland), and was used as a surrogate measure of muscle mass as previously demonstrated.^{14,15} Other weight loss specific questions included are: "what was your lowest weight in the last 5 years", "are you currently on a diet to lose weight", and "have you ever experienced involuntary weight loss (6 kg in 6 months, or 3 kg in one month)".

PHYSICAL ASSESSMENT

At baseline, participants were invited to visit one of twelve LifeLines Research sites to undergo a physical examination and a series of tests. Anthropometric measurements including height (cm), weight (kg), and body mass index (BMI) were conducted by nurses during the study visit. BMI was categorized in 2 groups (i.e., BMI <18.5 kg/m² and BMI ≥18.5 kg/m²).

STATISTICAL ANALYSIS

Statistics were performed with The Statistical Product and Service Solutions (SPSS; version 22, Released 2013, IBM Corp., Armonk, NY). Results were expressed as number of subjects (percentages) mean ± SD or median (IQR) for categorical, normally and non-normally distributed data, respectively. Chi-Square test, Independent samples t-test, and Mann Whitney U test were used as appropriate to compare characteristics between participants with and without RP. Binary logistic regression with the presence of definite RP (no/yes) as dependent variable was performed (presented as odds ratio (OR) and 95% CI) in order to assess the association between BMI, weight loss, and the presence of RP.

Given the fact that BMI and weight loss can cause problems of co-linearity if included together, these variables were included separately in the model. Variables that were statistically significant in the univariate analyses were further analyzed in multivariate logistic regression (enter method) to correct for potential confounders. Current literature has shown that BMI and weight loss might be influenced by daily caloric intake and muscle mass (creatinine was used as a surrogate of muscle mass) and therefore we decided to correct for these parameters.^{16–19} In addition, we also corrected for other known etiological factors of RP, such as age, known CTD, smoking, use of medication (i.e., beta-blocking agents, immunosuppressive drugs, and central acting sympathomimetic). In women, we additionally corrected for hormonal status (pre vs. post-menopausal, receiving hormonal contraception, receiving hormonal treatment other than contraception). All analyses were stratified for gender (effect modifier). P-values below 0.05 were considered as statically significant.

RESULTS

PATIENT CHARACTERISTICS

A total of 152020 participants were included in the Lifelines Cohort Study. The CTD questionnaire was completed by 93935 (61.8% of total, figure 1), with a mean age of 45.6 (± 12.9) years, of whom 56122 (59.7%) were women and 37813 (40.3%) men. Further characteristics of the responders can be found in table 1. Of the non-responders (participants who were sent the questionnaire but declined to complete, total n=58085), 32901 (56.5%) were women and mean age was 43.1 (± 13.4) years.

Figure 1. Overview of the reported discoloration of the hands. A total of 93,935 subjects completed the questionnaire, of whom 10.6% reported monophasic discoloration. Of the subjects who reported monophasic discoloration, 4.2% also reported biphasic or triphasic discoloration. CTD, connective tissue disease.

Table 1: Patient characteristics of the total group, participants with and without RP.

Patient characteristics	Total responders (n=93935)	Non-RP (n= 70037)	RP (n=3927)	*P-value
Gender, women, n (%)	56122 (59.7%)	39185 (55.9%)	3154 (80%)	<0.001
Age in years at baseline, median (IQR)	46 (37-54)	45 (37-53)	43 (33-50)	<0.001
Comorbidities, n (%)				
CTD (SLE, Sjögren, Systemic sclerosis)	40 (0.04%)	4 (0.006%)	4 (0.1%)	0.61
Use of medication, n (%)				
Beta-blockers	5408 (5.8%)	3674 (5.2%)	222 (5.7%)	0.27
Immunosuppressive drugs	2578 (2.7%)	1698 (2.4%)	166 (4.2%)	<0.001
Central acting sympathomimetic	317 (0.3%)	200 (0.3%)	25 (0.6%)	<0.001
Smoking behavior, n (%)				
Current smoker	17507 (18.6%)	12952 (18.5%)	749 (19.1%)	0.65
Anthropometric measurements				
Weight (kg), median (IQR)	78 (69-89)	79 (70-90)	70 (63-80)	<0.001
Length (cm), median (IQR)	174 (168-182)	175 (168-182)	172 (167-178)	<0.001
BMI in kg/m ² , median (IQR)	25.4 (23.1-28.1)	25.6 (23.4-28.4)	23.5 (21.4-26.1)	<0.001
Low body weight (BMI<18.5)	697 (0.8%)	372 (0.5%)	92 (2.3%)	<0.001
Nutritional information				
On a diet to lose weight, n (%)	5969 (6.4%)	4425 (6.3%)	241 (6.1%)	0.65
Lowest weight in the last 5 years, median (IQR)	72 (63-82)	73 (64-83)	65 (58-73)	<0.001
Involuntary weight loss (6 kg in in 6 months or 3 kg in one month), n (%)	2780 (3.0%)	1805 (2.6%)	207 (5.3%)	<0.001
Daily caloric intake (in kcal), median (IQR)	1888 (1484-2319)	1911 (1501-2341)	1798 (1431-2233)	<0.001
Low calorie intake (<1000 calories/per day)	8645 (9.2%)	8192 (11.7%)	453 (11.5%)	0.76
Daily intake of carbohydrates (g/day), median (IQR)	210 (163-2320)	213 (164-264)	206 (160-256)	<0.001
Low-carbohydrate diet, n (%)	3368 (3.6%)	2528 (3.6%)	126 (3.2%)	0.19
Daily intake of fat (g/day), median (IQR)	73 (54-94)	74 (55-94)	69 (51-89)	<0.001
Low-fat diet, n (%)	17355 (18.5%)	12934 (18.5%)	810 (20.6%)	0.001
Creatinine level (umol/l), median (IQR)	72 (64-81)	73 (65-82)	70 (63-77)	<0.001

Data are shown as n (%) or median (interquartile range). Abbreviations: CTD, connective tissue disease; SLE, systemic lupus erythematosus; BMI, body mass index. *The p-value depicts the difference between the non-Raynaud's group and the Raynaud's group.

PREVALENCE OF RAYNAUD'S PHENOMENON

Figure 2 shows the prevalence of RP in our cohort. In total, 3927 participants reported at least biphasic discoloration, and, thus were classified as definite RP. The overall prevalence rate of definite RP was found to be 4.2% [95% CI 4.1-4.4]. Regarding the age-specific prevalence rates, we observed that the prevalence of definite RP decreases with increasing age. Participants aged 18-20 years showed the highest prevalence rate (men 2.5% [1.3-4.7], women 10.2% [8.5-12.3], $p<0.001$), and prevalence was found to decline with age.

Figure 2. Prevalence (95% confidence intervals) of Raynaud's phenomenon (RP), in the different age groups, shown for men and women.

DIFFERENCES IN ANTHROPOMETRIC MEASUREMENTS AND DIETARY ASSESSMENT IN PARTICIPANTS WITH AND WITHOUT RP

Subjects with RP had a lower current weight, as compared to subjects without RP ($p<0.001$). In addition, low body weight (i.e., $BMI<18.5$) was more frequently found in subjects with RP as compared to subjects without RP (RP 2.3%, non-RP 0.5%, $p<0.001$). The lowest (self-measured) weight reported in the last 5 years was 8 kg lower in subjects with RP, as compared to subjects without RP (RP median 65 kg (IQR 58-73), non-RP 73 kg (64-83), $p<0.001$). In addition, subjects with RP more frequently reported to have suffered from involuntary weight loss (RP 5.3%, non-RP 2.6%, $p<0.001$). With regard to the nutritional information, subjects with RP did have a significantly lower daily caloric intake, as compared to subjects without RP (RP 1798 calories per day, non-RP 1911 calories per day, $p<0.001$). However, the number of subjects who were currently on a diet to lose weight did not differ between groups ($p=0.645$). Creatinine levels, as a surrogate of muscle mass, were also found to be significantly lower in subjects with RP (RP median 70 (IQR 63-77), non-RP: 73 (65-82), $p<0.001$).

Figure 3. (A) Univariate and (B) multivariate analysis of the relationship between the presence of Raynaud's phenomenon and low body weight, involuntary weight loss, body weight (kg), low-carbohydrate diet, low-fat diet, and low-calorie intake, shown for men and women. In the multivariate analysis we corrected for creatinine level (as a surrogate measure of muscle mass), daily caloric intake, age, known connective tissue disease, smoking, and use of medication (i.e., beta-blocking agents, immunosuppressive drugs, and central-acting sympathomimetics). In women, we additionally corrected for hormonal status (pre- vs post-menopausal, receiving hormonal contraception, receiving hormonal treatment other than contraception).

THE ASSOCIATION BETWEEN BMI AND RAYNAUD'S PHENOMENON

The interaction term between BMI and gender was found to be statistically significant ($p=0.01$), which indicates that gender is an effect modifier. Therefore, the univariate and multivariate analyses were stratified for gender and presented in figure 3A and 3B, respectively.

In men, the univariate analysis showed that weight (kg), low body weight (BMI<18.5), and involuntary weight loss, were significantly associated with the presence of RP. The multivariate analyses revealed that, after correction for potential confounders, these variables remained significantly associated with the presence of RP (figure 3B) (weight OR 0.96 [95% CI 0.96-0.97], low body weight OR 5.55 [95% CI 2.82-10.93], involuntary weight loss OR 1.32 [95% CI 1.17-1.48]).

In women, the univariate analyses showed that weight (kg), low body weight, involuntary weight loss, low-carbohydrate diet, and low-fat diet were significantly associated with the presence of RP. After adjustment for potential confounders, these variables, with the exception of low-carbohydrate diet, remained significantly associated with the presence of RP (weight OR 0.97 [0.96-0.97], low body weight OR 3.14 [2.40-4.10], involuntary weight loss OR 1.31 [1.20-1.44], and low-fat diet OR 1.27 [1.15-1.41]).

DISCUSSION

To the best of our knowledge, this is largest study to investigate the association between low body weight, involuntary weight loss, dietary restriction and RP in the general population. We clearly demonstrated that low body weight and prior involuntary weight loss are significantly associated with an increased risk of RP in both men and women. In addition, low-fat diet was found to be associated with RP in women, but not in men. These associations remained present after correction for known etiological factors associated with RP and other factors that might influence body weight. Conversely, low-calorie intake (<1000 calories per day) was not found to be associated with RP. Collectively, our results underline the importance of body weight and nutritional issues as easily overlooked associates of RP.

Subjects with RP had a lower daily caloric intake (approximately 100 calories lower), as compared to subjects without RP. Interestingly, the number of subjects who were currently on a diet to lose weight did not differ between groups. In addition, we demonstrated that underweight subjects, and subjects with involuntary weight loss have an increased odds of RP, independently of age, current smoking, creatinine level (as a surrogate measure of muscle mass), daily caloric intake, and hormonal status (in women). However, studies focusing on the relationship between BMI and RP are very limited, and, therefore, the exact causal relationship remains poorly understood. For instance, it could be hypothesized that substantial weight loss (or a BMI<18.5) increases a person's susceptibility to RP. A possible explanation is that weight loss may lead to a loss of subcutaneous and

perivascular adipose tissue, and subsequently increases the susceptibility to vasospastic events. In support, it has been shown that perivascular adipose tissue alters the balance between endothelium-dependent vasodilator and vasoconstrictor substances (such as nitric oxide and endothelin-1),²⁰ which further underlines the importance of BMI (and more importantly recent weight loss) in the occurrence of RP. In addition, a recently conducted study showed that low body weight was also associated with an increased risk of cardiovascular disease (CVD).²¹ The authors stated that the increased risk of CVD could potentially be explained by the poor nutritional status and the decline in muscle mass in underweight subjects.²¹ In addition, it was previously reported that subjects with low muscle mass were at increased mortality risk.²² Although only a surrogate marker of muscle mass, the current study found that subjects with RP had lower levels of creatinine. Therefore, it could be hypothesized that subjects with low muscle mass may also have a higher susceptibility to develop RP. Collectively, these results underline the importance of investigating body composition to differentiate the separate contribution of muscle and fat mass as a possible contributing factor for RP. Given the fact that an increase in body weight was found to be associated with a slightly decreased odds of RP, future studies should also focus on the potentially protective effects of fat.

In our study the association of low body weight and involuntary weight loss with the presence of RP remained present after correction for hormonal status (including hormonal treatment other than contraceptives) in women. This indicates that the causal pathway in which BMI affects RP is not solely mediated by hormonal factors in women, and that other unknown factors might have a greater role in this pathway. Interestingly, we found that low-fat diet was associated with an increased risk of RP in women. This could indicate a poor nutritional status.²³ However, studies investigating dietary restrictions in RP do not exist and, therefore, the clinical relevance of this association is merely speculative.

This is the largest epidemiological study to assess the prevalence of RP in the general population. Garner et al. (2015) conducted a meta-analysis and reported an overall prevalence of definite RP ranging between 1.6%-7.2%.²⁴ This is in accordance with our findings, however, other studies have reported much lower and also higher prevalence rates.^{25,26} The large variation in prevalence rates could be explained by regional differences as previous studies have clearly demonstrated that the prevalence of RP is increased in colder regions of the world.²⁷ In support, Brand et al. (1997) and Fraenkel et al. (1999) reported prevalence's of 7.2% and 7.8% in Boston (USA).^{1,28} In countries with a warmer climate, the prevalence of RP was estimated to range between 2-6%.²⁹⁻³¹ The variation in reported prevalence rates can also be explained by differences in definition of RP. For instance, only 39% of the previously conducted studies investigating the prevalence of RP used a precise definition of RP.²⁴ Without a universally agreed upon definition, studies may over- or underestimate the true prevalence of RP.^{32,33} The definition used in the current study was comparable to several previously conducted studies,^{26,33,34} and is in accordance with the definition of the UK Scleroderma group.⁹

The limitations of this study include the cross-sectional design, which does not allow investigation of causality. In addition, the primary outcome parameters were solely based on questionnaires which could have introduced a bias into our paper. However, this is a population based study, which was designed to answer multiple research questions, and was not only set-up for the purpose of the current paper. Since a large sample of the general population of the Northern parts of the Netherlands was included, the clinical information that was feasible to be assessed in such a large sample was of course limited. In line with this limitation, more specific assessment of CTD specific data (including nailfold videocapillaroscopy) was not feasible at that point. Furthermore, the self-reported nature of the CTD questionnaire might lead to an overestimation of the prevalence of RP. However, the CTD questionnaire used in the current study was previously validated, and was found to have a high sensitivity and specificity for screening large populations.⁷ Furthermore, the CTD questionnaire was completed within 3 years following the baseline assessment. Although variables assessed at baseline (e.g., body weight) could have changed over time, we believe that given the large sample size of this study this would only minimally influence our results. In addition, occupational exposure was not included in the current study. Therefore, the role of environmental factors (e.g., use of vibrating tools) remains unknown. Nevertheless, our study provides crucial information on the true burden of RP in the general population and its relationship with low body weight. Our study will raise awareness among physicians to pay more attention to body weight and nutritional issues as possible treatable causes of RP.

In conclusion, this large population-based cohort study clearly demonstrated that low body weight and prior involuntary weight loss are significantly associated with an increased risk of RP in both men and women. The current study underlines the importance of these possibly treatable associates of RP, and further emphasizes that BMI and weight changes should be carefully assessed and monitored in subjects presenting with RP.

REFERENCES

1. Fraenkel L, Zhang Y, Chaisson CE, et al. Different factors influencing the expression of Raynaud's phenomenon in men and women. *Arthritis Rheum.* 1999;42(2):306-310. doi:10.1002/1529-0131(199902)42:2<306::AID-ANR13>3.0.CO;2-G
2. Herrick AL. Pathogenesis of Raynaud's phenomenon. *Rheumatology.* 2005;44(5):587-596. doi:10.1093/rheumatology/keh552
3. Yucel B, Kubat Uzum A, Ozbey N, Kamali S, Yager J. Anorexia Nervosa and Raynaud's Phenomenon: A Case Report. *Int J Eat Disord.* 2007;40(2):762-765. doi:10.1002/eat
4. Sachs K V, Harnke B, Mehler PS, Krantz MJ. Cardiovascular complications of anorexia nervosa: A systematic review. *Int J Eat Disord.* 2016;49(3):238-248. doi:10.1002/eat.22481
5. De Martinis M, Sirufo MM, Ginaldi L. Raynaud's phenomenon and nailfold capillaroscopic findings in anorexia nervosa. *Curr Med Res Opin.* 2018;34(3):547-550. doi:10.1080/03007995.2017.1417828
6. Stolk RP, Rosmalen JGM, Postma DS, et al. Universal risk factors for multifactorial diseases: LifeLines: A three-generation population-based study. *Eur J Epidemiol.* 2008;23(1):67-74. doi:10.1007/s10654-007-9204-4
7. Karlson EW, Sanchez-Guerrero J, Wright EA, et al. A connective tissue disease screening questionnaire for population studies. *Ann Epidemiol.* 1995;5(4):297-302. doi:10.1016/1047-2797(94)00096-C
8. Karlson EW, Costenbader KH, McAlindon TE, et al. High sensitivity, specificity and predictive value of the Connective Tissue Disease Screening Questionnaire among urban African-American women. *Lupus.* 2005;14(10):832-836. doi:10.1191/0961203305lu2227oa
9. Brennan P, Silman A, Black C, et al. Validity and reliability of three methods used in the diagnosis of Raynaud's phenomenon. *Rheumatology.* 1993;32(5):357-361. doi:10.1093/rheumatology/32.5.357
10. Vinke PC, Corpeleijn E, Dekker LH, Jacobs DR, Navis G, Kromhout D. Development of the food-based Lifelines Diet Score (LLDS) and its application in 129,369 Lifelines participants. *Eur J Clin Nutr.* 2018;72(8):1111-1119. doi:10.1038/s41430-018-0205-z
11. Brinkworth GD, Noakes M, Buckley JD, Keogh JB, Clifton PM. Long-term effects of a very-low-carbohydrate weight loss diet compared with an isocaloric low-fat diet after 12 mo. *Am J Clin Nutr.* 2009;90(1):23-32. doi:10.3945/ajcn.2008.27326
12. Tobias DK, Chen M, Manson JAE, Ludwig DS, Willett W, Hu FB. Effect of low-fat diet interventions versus other diet interventions on long-term weight change in adults: A systematic review and meta-analysis. *Lancet Diabetes Endocrinol.* 2015;3(12):968-979. doi:10.1016/S2213-8587(15)00367-8
13. Samaha FF, Iqbal N, Seshadri P, et al. A low-carbohydrate as compared with a low-fat diet in severe obesity. *N Engl J Med.* 2003;348(21):2074-2081. doi:10.1056/NEJMoa022637
14. Schutte JE, Longhurst JC, Gaffney FA, Bastian BC, Blomqvist CG. Total plasma creatinine: An accurate measure of total striated muscle mass. *J Appl Physiol Respir Environ Exerc Physiol.* 1981;51(3):762-766.
15. Thongprayoon C, Cheungpasitporn W, Kashani K. Serum creatinine level, a surrogate of muscle mass, predicts mortality in critically ill patients. *J Thorac Dis.* 2016;8(5):E305-E311. doi:10.21037/jtd.2016.03.62
16. Soini S, Mustajoki P, Eriksson JG. Lifestyle-related factors associated with successful weight loss. *Ann Med.* 2015;47(2):88-93. doi:10.3109/07853890.2015.1004358
17. Pansini F, Cervellati C, Guariento A, et al. Oxidative stress, body fat composition, and endocrine status in pre- and postmenopausal women. *Menopause.* 2008;15(1):112-118. doi:10.1097/gme.0b013e318068b285
18. Jakubowicz D, Barnea M, Wainstein J, Froy O. High Caloric intake at breakfast vs. dinner differentially influences weight loss of overweight and obese women. *Obesity.* 2013;21(12):2504-2512. doi:10.1002/oby.20460

19. Wysokiński A, Sobów T, Kłoszewska I, Kostka T. Mechanisms of the anorexia of aging—a review. *Age (Omaha)*. 2015;37(4):9821. doi:10.1007/s11357-015-9821-x
20. Houben AJ, Eringa EC, Jonk AM, Serne EH, Smulders YM, Stehouwer CD. Perivascular Fat and the Microcirculation: Relevance to Insulin Resistance, Diabetes, and Cardiovascular Disease. *Curr Cardiovasc Risk Rep*. 2012;6(1):80-90. doi:10.1007/s12170-011-0214-0
21. Park D, Lee JH, Han S. Underweight: Another risk factor for cardiovascular disease? *Med (United States)*. 2017;96(48):e8769. doi:10.1097/MD.00000000000008769
22. Srikanthan P, Horwich TB, Tseng CH. Relation of Muscle Mass and Fat Mass to Cardiovascular Disease Mortality. *Am J Cardiol*. 2016;117(8):1355-1360. doi:10.1016/j.amjcard.2016.01.033
23. Siguel EN, Lerman RH. Role of essential fatty acids: dangers in the US Department of Agriculture dietary recommendations ("pyramid") and in low-fat diets. *Am J Clin Nutr*. 1994;60(6):973-974.
24. Garner R, Kumari R, Lanyon P, Doherty M, Zhang W. Prevalence, risk factors and associations of primary Raynaud's phenomenon: systematic review and meta-analysis of observational studies. *BMJ Open*. 2015;5(3):e006389. doi:10.1136/bmjopen-2014-006389
25. Bartelink ML, Wollersheim H, van de Lisdonk E, Spruijt R, van Weel C. Prevalence of Raynaud's phenomenon. *Neth J Med*. 1992;41(3-4):149-152.
26. Purdie G, Harrison A, Purdie D. Prevalence of Raynaud's phenomenon in the adult New Zealand population. *N Z Med J*. 2009;122(1306):55-62.
27. Maricq HR, Carpentier PH, Weinrich MC, et al. Geographic variation in the prevalence of Raynaud's phenomenon: a 5 region comparison. *J Rheumatol*. 1997;24(5):879-889.
28. Brand FN, Larson MG, Kannel WB, McGuirk JM. The occurrence of Raynaud's phenomenon in a general population: The Framingham Study. *Vasc Med*. 1997;2:296-301.
29. Román Ivorra JA, González Perales JL, Fernández Carballido C, Graña J, Torres MJ. Prevalence of Raynaud's phenomenon in general practice in the East of Spain. *Clin Rheumatol*. 2001. doi:10.1007/s100670170076
30. De Angelis R, Salaffi F, Grassi W. Raynaud's phenomenon: Prevalence in an Italian population sample. *Clin Rheumatol*. 2006;25:506-510. doi:10.1007/s10067-005-0077-1
31. Onbaşı K, Şahin İ, Onbaşı O, Üstün Y, Koca D. Raynaud's phenomenon in a healthy Turkish population. *Clin Rheumatol*. 2005. doi:10.1007/s10067-004-1045-x
32. Herrick AL. The pathogenesis, diagnosis and treatment of Raynaud phenomenon. *Nat Rev Rheumatol*. 2012;8(8):469-479. doi:10.1038/nrrheum.2012.96
33. Mavarakis E, Patel F, Kronenberg DG, et al. International consensus criteria for the diagnosis of Raynaud's phenomenon. *J Autoimmun*. 2014. doi:10.1016/j.jaut.2014.01.020
34. Wigley FM. Clinical practice. Raynaud's Phenomenon. *N Engl J Med*. 2002;347(13):1001-1008.

CHAPTER

Nailfold capillaroscopy is associated with BMI but not with serum levels of adiponectin in subjects with Raynaud's phenomenon

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ABSTRACT

Objectives: To investigate the occurrence of nailfold capillary microscopy (NCM) abnormalities in RP patients in different BMI categories, and whether the vasoprotective mediator adiponectine, which is produced by fat tissue, is related to NCM.

Methods: NCMs were retrospectively assessed in 351 RP patients, without an underlying systemic disease. Patients were divided by BMI (kg/m^2) category (BMI<18.5, BMI 18.5-24.99, BMI $\geq$ 25). NCM was quantitatively assessed. A standardized cooling experiment was performed in 152 patients, and serum levels of adiponectin were measured in an unselected subgroup of 36 patients.

Results: Lowest count of capillaries (BMI<18.5: 20.9 ($\pm$ 3.2), BMI 18.5-24.99: 22.2 ($\pm$ 2.7), BMI $\geq$ 25: 23.2 ($\pm$ 2.0), $p<0.001$), highest counts of dilated capillaries (BMI<18.5: 5.1 ($\pm$ 4.0), BMI 18.5-24.99: 4.2 ($\pm$ 3.7), BMI $\geq$ 25: 2.6 ($\pm$ 3.1), $p=0.001$), and giant capillaries (BMI<18.5: 1.0 ($\pm$ 2.2), BMI 18.5-24.99: 0.5 ($\pm$ 1.2), BMI $\geq$ 25: 0.2 ($\pm$ 1.1), $p=0.001$) were found in underweight RP patients. Patients with a BMI $\geq$ 25 kg/m^2 showed a shorter mean recovery time as compared to the other groups (BMI< 18.5: 6.01 min ($\pm$ 3.4), BMI 18.5-24.99: 5.81 min ($\pm$ 3.6), BMI $\geq$ 25: 4.23 min ($\pm$ 3.3), $p=0.029$). No associations were found between NCM and serum levels of adiponectin.

Conclusion: These findings indicate that microvascular abnormalities are associated with BMI and occur independently of a connective tissue disease. Although we did not observe a relationship between adiponectin and NCM in a small subset, these preliminary results might suggest a protective role of (perivascular) fat in Raynaud's phenomenon and warrants future studies.

INTRODUCTION

Raynaud's phenomenon (RP) is a frequently occurring disorder, characterized by an episodic discoloration of the extremities in response to cold exposure or emotional stress. RP can be either primary (idiopathic), which often occurs in young females, or secondary to many diseases and conditions.¹⁻³ Although the exact pathogenesis is incompletely understood, BMI is thought to play an important role in the pathogenesis of RP.

A recently conducted large cohort study in a Danish population (n=75008) showed that the risk of RP increases by each decrease of a body mass index (BMI) unit.⁴ Microvascular changes, assessed with nailfold capillary microscopy (NCM), were also found to co-occur with certain eating disorders (e.g., anorexia nervosa), in the absence of an underlying auto-immune disease.⁵ Mueller et al. (2016) have demonstrated that the presence of an abnormal NCM is associated with poor survival in RP patients without previously known connective tissue disease (CTD).⁶ The presence of microvascular changes are also associated with the severity of RP, as documented with cooling plethysmography, indicating that mechanisms (other than underlying CTDs) may play an important role.⁷

Some have implicated adipocytes surrounding the cutaneous blood vessels (perivascular adipose tissue; PVAT) to play an important role in the pathogenesis of RP, as these cells may contribute to the thermogenic activity and maintenance of intravascular temperatures in response to cold stimuli.⁸ More particularly, adiponectin, which can be produced by PVAT and is believed to be an important vasoactive regulator,⁹ was previously proposed to protect against vascular dysfunction.¹⁰ Although a reduction of subcutaneous fat and PVAT might increase a patient's susceptibility to develop RP, the exact role of adiponectin in the pathogenesis of RP still remains not well clarified.

The current study aims to investigate the occurrence of NCM abnormalities in RP patients in several BMI categories (low BMI, normal BMI, and high BMI). In addition, we will also investigate the difference in digital perfusion during a standardized cooling and recovery experiment in relation to BMI. Lastly, in a sub-group analysis, we will explore the association between NCM and serum level of adiponectin.

MATERIALS AND METHODS

PARTICIPANTS

NCM results and clinical characteristics were retrospectively assessed in patients with RP who presented between November 2008 to August 2013. Laboratory tests (e.g., serological tests) and, in the case of suspected secondary RP, functional test (e.g., pulmonary function tests, esophageal scintigraphy, high-resolution CT and cardiac ultrasound) were also obtained. The diagnosis of RP was confirmed by the treating physician, and patients were included when no clinical (i.e., negative ANA and no signs of organ involvement) evidence of an underlying connective tissue disease was found after at least 4 years follow-up. This study was approved by the local medical ethics committee (METc 2015/175, Groningen, The Netherlands). Given that the study does not fall under the Dutch law of medical research in humans, the local ethics committee has provided exemption from written informed consent.

CLINICAL AND LABORATORY ASSESSMENTS

Patient characteristics (including sex, age, body weight and height) were collected. Patients were divided by BMI category: underweight ($\text{BMI} < 18.5 \text{ kg/m}^2$), healthy weight ($\text{BMI} 18.5\text{--}24.99 \text{ kg/m}^2$), and overweight ($\text{BMI} \geq 25 \text{ kg/m}^2$). NCM of the middle and ring finger (of both hands) was assessed in all patients as described previously.^{7,11–14} All images of nailfolds were taken at 180x magnification. The number of capillaries (per 3mm) was counted for each finger, and the mean number of capillaries per finger per subject was derived. As the set-up used in this study could not measure capillary dimension, dilated and giant capillaries were only judged visually by an experienced observer. Patterns were deemed abnormal if the mean capillary count was < 18 ,¹⁵ or mean number of dilated capillaries per finger was > 3 , or any giant capillaries were observed.

A standardized cooling and recovery experiment was performed in 152 patients ($n=22$ for $\text{BMI} < 18.5 \text{ kg/m}^2$; $n=87$ for $18.5\text{--}24.99 \text{ kg/m}^2$; $n=43$ for $\geq 25 \text{ kg/m}^2$) as described previously.⁷ In short, the left hand of the subject, with photo-electric plethysmography (PPG) sensors attached to all five fingers, was submerged in water to the level of the radiocarpal joint. The cooling procedure started with water at 33°C and every 4 minutes the water was cooled by 3°C . After the cooling steps, the hand was taken out of the water, and recovery of perfusion was tracked for 10 min. Perfusion was recorded during the last 15 seconds of every 4 minutes during cooling, and during the last 15 seconds of every minute during recovery. Mean ischemic time was defined as the mean time of perfusion loss during cooling and recovery, whereas mean recovery time was defined as the mean time of perfusion loss during recovery.

Serum levels of adiponectin were measured in a sub-group consisting of 36 patients ($\text{BMI} < 18.5 \text{ kg/m}^2$ $n=8$; $\text{BMI} 18.5\text{--}24.99 \text{ kg/m}^2$ $n=21$; $\text{BMI} \geq 25 \text{ kg/m}^2$ $n=7$). Unfortunately, serum was not available in more patients. Levels of adiponectin were measured using commercially available ELISA kits (R&D Systems, USA), and calculated according to the manufacturer's recommendations.

STATISTICAL ANALYSIS

Statistics were performed with The Statistical Product and Service Solutions (SPSS; version 22, Released 2013, IBM Corp., Armonk, NY). Non-parametric tests (χ^2 , Mann-Whitney U or Kruskal Wallis) were used in case of non-normally distributed data to determine group differences. Pairwise comparisons were performed, adjusted by the Bonferroni correction for multiple testing. Correlations between variables were determined by either Pearson or Spearman correlation coefficients. A p-value below 0.05 was considered significant.

RESULTS

A total of 351 patients with RP were enrolled in the present study. None of the patients showed signs of an underlying connective tissue disease. The median age was 40.6 (IQR 24.6-52.9) with a male/female ratio of 111/240. Forty-five patients were underweight (BMI <18.5 kg/m²), 219 had a healthy weight (BMI 18.5-24.99 kg/m²), and 87 patients were overweight (BMI ≥25 kg/m²) (Table 1).

Table 1: Patients characteristics displayed per body mass index (BMI) (kg/m²) category.

	BMI <18.5 N=45	BMI 18.5-24.99 N=219	BMI ≥25 N=87	P-value
Age (median, range)	22.5 (17.5-44.9)	38.1 (24.0-52.2)	49.7 (39.6-58.9)	<0.001
Female gender, n(%)	34 (75.6%)	160 (71.7%)	49 (55.7%)	0.01
Smoking (previous and current), n(%)	13 (28.9%)	79 (35.4%)	17 (19.3%)	0.02

ASSOCIATION BETWEEN BMI AND NCM

The observed NCM abnormalities are displayed per group in Figure 1. The mean number of capillaries for all patients was 22.3 per finger (SD± 2.7) and patients with a BMI<18.5 kg/m² showed significantly lower counted capillaries (20.9 ± 3.2) compared to the other groups (BMI 18.5-24.99 kg/m²: 22.2 (± 2.7), BMI ≥25 kg/m²: 23.2 (± 2.0), p<0.001). Underweight RP patients also had the highest mean count of dilated capillaries (BMI<18.5 kg/m²: 5.1 (± 4.0), BMI 18.5-24.99 kg/m²: 4.2 (±3.7), BMI ≥25 kg/m²: 2.6 (±3.1), p=0.001), and giant capillaries (BMI<18.5 kg/m²: 1.0 (± 2.2), BMI 18.5-24.99 kg/m²: 0.5 (±1.2), BMI ≥25 kg/m²: 0.2 (±1.1), p=0.001). Post-hoc analysis of the pairwise comparisons is shown in figure 1.

Figure 1: Percentage of patients with abnormalities on nailfold capillary microscopy, depicted for each BMI category. P-value represents the post-hoc analysis, adjusted by the Bonferroni correction for multiple testing. Abbreviations: NCM= nailfold capillary microscopy; BMI= body mass index.

ASSOCIATION BETWEEN BMI AND DIGITAL ISCHEMIA

The mean number of digits with normal perfusion for each BMI group, assessed with PPG during cooling and recovery experiment, are depicted in Figure 2. The mean ischemic time did not significantly differ between groups (17.82 min ($\pm$ 9.59) for BMI < 18.5 kg/m²; 17.93 min ($\pm$ 8.74) for BMI 18.5-24.99 kg/m²; 18.64 min ($\pm$ 8.64) for BMI $\geq$ 25 kg/m², $p=0.93$). However, there was a significant difference between groups in mean recovery time (6.0 min ($\pm$ 3.4) for BMI < 18.5 kg/m²; 5.8 min ($\pm$ 3.6) for BMI 18.5-24.99 kg/m²; 4.2 min ($\pm$ 3.3) for BMI $\geq$ 25 kg/m², $p=0.03$). Post-hoc analysis only revealed a significant difference in mean recovery time between BMI $\geq$ 25 kg/m² and BMI 18-24.99 kg/m² ($p=0.04$).

Figure 2: Mean number of digits with normal perfusion assessed with photoelectric plethysmography during stepwise cooling and recovery. Abbreviations: BMI= body mass index.

ASSOCIATION BETWEEN ADIPONECTIN, BMI AND NCM (EXPLORATORY SUB-STUDY)

Adiponectin levels were not significantly different between groups (median [IQR], BMI < 18.5: 12806.6 ng/ml [9002.7-16876.2], BMI 18.5-24.99: 13031.6 ng/ml [10157.3-15738.7], BMI $\geq$ 25: 6455.5 ng/ml [5492.3-9361.4], $p=0.15$, figure 3).

Mean capillary count ($r=0.26$, $p<0.001$), mean number of dilated capillaries ($r=-0.22$, $p<0.001$), and mean number of giant capillaries ($r=-0.23$, $p<0.001$) were associated with BMI. No associations were found between NCM and serum level of adiponectin (mean capillary count $r=0.03$, $p=0.88$; mean number of dilated capillaries $r=0.07$, $p=0.67$; mean number of giant capillaries $r=-0.16$, $p=0.34$).

Figure 3: Serum level of adiponectin depicted per BMI category. Abbreviations: BMI= body mass index.

DISCUSSION

To the best of our knowledge, this is the first study to investigate potential mechanisms of Raynaud's phenomenon in relation to BMI. The main finding of the current study was that underweight RP patients more frequently showed NCM abnormalities. In addition, patients with a higher BMI showed a significantly shorter mean recovery time as compared to the other groups. In our sub-group analysis we were unable to demonstrate any association between NCM and serum levels of adiponectin. Our findings indicate that the occurrence of microvascular abnormalities in underweight RP patients can occur independently of the presence of a connective tissue disease, and have to be attributed to other mechanisms. The exact role of adiponectin has to be further investigated in a larger study.

Despite the extensive research on this subject, the exact pathogenesis of RP still remains incompletely understood. The current understanding of this rather complex phenomena is that the imbalance between vasoconstriction and vasodilatation is purely functional and that structural vascular changes do not occur in PRP patients.² In our current cohort, patients in the BMI category <18.5 kg/m² not only had a lower capillary count, but more frequently showed dilated capillaries and giant capillaries. In addition, BMI was found to be positively associated with mean capillary count and negatively associated with the occurrence of dilated and giant capillaries. Bukhari et al. (1996) have previously compared NCM of PRP patients, SSc patients, and healthy controls and found capillary dimensions to be increased and capillary density to be decreased, even in PRP patients.¹⁶ Therefore, we postulate that vascular abnormalities can indeed occur in underweight RP patients

without an underlying connective tissue disease, and, thereby, low body weight may be considered as an additional risk factor of RP.^{1,17} Further supporting our postulation, Klein-Weigel et al. (2004) have reported that dilated efferent capillary loops and venules were more frequently present in symptomatic anorexia nervosa patients compared with non-symptomatic patients.⁵ Another study reported that anorexia nervosa is indeed associated with peripheral vascular abnormalities (e.g., dysregulation of peripheral vasoconstriction/vasodilation, acrocyanosis, arterial vasospasm).¹⁸ Palova et al. (2013) have demonstrated that not only flow-mediated vasodilatation is decreased in patients with anorexia, but also that this abnormality improves following renourishing.¹⁹ Therefore, a compensatory heat conserving, selective peripheral vasoconstriction is a plausible and perhaps reversible cause of RP in these underweight patients. In a recently conducted study from our center, it was found that the degree of vasospasm and ischemia, was positively associated with the NCM.⁷ Therefore, our observed NCM abnormalities may also be explained as a compensation for the reoccurring vasospasms, and perhaps the occurrence of prolonged attacks, due to the higher sensitivity to cold temperatures.

Interestingly, underweight RP patients in our study were substantially younger compared to the other two groups. Although not knowing the exact cause of the low BMI in our cohort, we do know that eating disorders affect between 1-10% of adolescent and college-age women.²⁰ Therefore, we have to take into account the possibility that presence of underlying eating disorders may also have influenced our results.

Despite the fact that the association between RP and BMI has been previously reported,²¹ the underlying mechanisms are less frequently discussed. Our findings indicate that patients with a higher BMI have a significantly shorter mean recovery time compared to underweight patients and patients with a healthy BMI. This finding might suggest a protective role of fatty tissue against vasospastic events. In support, it was previously reported that flow-mediated dilation improved after long-term estrogen replacement therapy in postmenopausal women with secondary RP.²² As estrogen levels may be increased in obese patients, due to peripheral aromatization of testosterone to estrogen,²³ this finding sheds light on the possible protective factor of an increased BMI in RP.

A decrease in perivascular adipose tissue may also play an important role in the occurrence of microvascular abnormalities in underweight RP patients. For instance, it has been shown that perivascular adipose tissue alters the balance between endothelium-dependent vasodilator and vasoconstrictor substances (such as nitric oxide and endothelin-1), which may be mediated by leptin and reactive oxygen species.²⁴ Adiponectin, which can be produced by PVAT, is associated with several vasoprotective effects including, but not limited to, increased production of endothelial NO,⁹ and, therefore, may also play an important role in the occurrence of microvascular abnormalities in underweight patients. Although a previously conducted study showed that adiponectin was negatively associated with BMI in patients with anorexia nervosa and bulimia nervosa,²⁵ our study was unable to demonstrate differences between BMI groups in serum levels of adiponectin. A possible explanation might be that adiponectin is only altered after extreme weight

loss, and is not altered in constitutionally thin subjects. However, given the fact that the sample size in our sub-group analysis was small, future studies should further investigate the role of adiponectin in underweight RP patients.

A limitation of this retrospective study is that we only assessed NCM in 4 fingers and not in 8, which was common practice in our center at the time of data collection. Despite this limitation, we were able to demonstrate clear differences between BMI groups and we believe that the inclusion of additional fingers would not have changed the results. In addition, other factors that could have potentially influenced BMI (i.e., decreased muscle mass) were not investigated in the current study. Since this was a retrospective study, serum samples available for analysis were limited. Therefore, the conclusion on adiponectin levels should be considered preliminary. We have assessed NCM only once per patient, therefore, possibly missing NCM changes over time, which could indicate progression. However, as these patients did not have signs of an underlying systemic disease (e.g., organ involvement and serological evidence) at baseline and no signs of an underlying disease developed over a follow-up period of at least 4 years, progression to a systemic disease remains very unlikely.

In conclusion, our findings indicate that microvascular abnormalities are inversely associated with BMI and occur independently of a connective tissue disease. The role of BMI in RP severity is further underlined by the shorter mean recovery time after cold challenge in patients with a high BMI. Although we did not observe a relationship between adiponectin and NCM, we believe the role of (perivascular) fat, as well as other potential body weight related mechanisms in subjects with RP, deserve further study.

REFERENCES

1. Fraenkel L, Zhang Y, Chaisson CE, et al. Different factors influencing the expression of Raynaud's phenomenon in men and women. *Arthritis Rheum.* 1999;42(2):306-310. doi:10.1002/1529-0131(199902)42:2<306::AID-ANR13>3.0.CO;2-G
2. Herrick AL. Pathogenesis of Raynaud's phenomenon. *Rheumatology.* 2005;44(5):587-596. doi:10.1093/rheumatology/keh552
3. Flavahan NA. A vascular mechanistic approach to understanding Raynaud phenomenon. *Nat Rev Rheumatol.* 2015;11(3):146-158. doi:10.1038/nrrheum.2014.195
4. Harpsøe MC, Basit S, Andersson M, et al. Body mass index and risk of autoimmune diseases: A study within the Danish National Birth Cohort. *Int J Epidemiol.* 2014;43(3):843-855. doi:10.1093/ije/dyu045
5. Klein-Weigel P, Rein P, Kronenberg F, et al. Microcirculatory assessment of vascular acrosyndrome in anorexia nervosa and analysis of manifestation factors. *J Psychosom Res.* 2004;56(1):145-148. doi:10.1016/S0022-3999(03)00381-7
6. Mueller M, Gschwandtner ME, Gamper J, et al. Relation of nailfold capillaries and autoantibodies to mortality in patients with raynaud phenomenon. *Circulation.* 2016;133(5):509-517. doi:10.1161/CIRCULATIONAHA.115.017816
7. van Roon AM, Smit AJ, van Roon AM, Bootsma H, Mulder DJ. Digital ischaemia during cooling is independently related to nailfold capillaroscopic pattern in patients with Raynaud's phenomenon. *Rheumatol (United Kingdom).* 2016;55(6):1083-1090. doi:10.1093/rheumatology/kew028
8. Chang L, Villacorta L, Li R, et al. Loss of perivascular adipose tissue on peroxisome proliferator-activated receptor- γ deletion in smooth muscle cells impairs intravascular thermoregulation and enhances atherosclerosis. *Circulation.* 2012;126(9):1067-1078. doi:10.1161/CIRCULATIONAHA.112.104489
9. Zhu W, Vanhoutte PM, Lam KSL, Cheng KKY, Xu A. Vascular effects of adiponectin: molecular mechanisms and potential therapeutic intervention. *Clin Sci.* 2008;114(5):361-374. doi:10.1042/cs20070347
10. Sena CM, Pereira A, Fernandes R, Letra L, Seica RM. Adiponectin improves endothelial function in mesenteric arteries of rats fed a high-fat diet: role of perivascular adipose tissue. *Br J Pharmacol.* 2017;174:3514-3526. doi:10.1111/bph.13756
11. Maricq HR, LeRoy EC, D'Angelo WA, et al. Diagnostic potential of in vivo capillary microscopy in scleroderma and related disorders. *Arthritis Rheum.* 1980;23(2):183-189. doi:10.1002/art.1780230208
12. Cutolo M, Pizzorni C, Tuccio M, et al. Nailfold videocapillaroscopic patterns and serum autoantibodies in systemic sclerosis. *Rheumatology.* 2004;43(6):719-726. doi:10.1093/rheumatology/keh156
13. Houtman PM, Kallenberg CG, Wouda AA, The TH. Decreased nailfold capillary density in Raynaud's phenomenon: a reflection of immunologically mediated local and systemic vascular disease? *Ann Rheum Dis.* 1985;44:603-609. doi:10.1136/ard.44.9.603
14. Cutolo M, Sulli A, Pizzorni C, Accardo S. Nailfold videocapillaroscopy Assessment of Microvascular Damage in Systemic Sclerosis. *Journal Rheumatol.* 2000;27:155-160.
15. Houtman PM, Kallenberg CGM, Fidler V, Wouda AA. Diagnostic significance of nailfold capillary patterns in patients with Raynaud's phenomenon. An analysis of patterns discriminating patients with and without connective tissue disease. *J Rheumatol.* 1986;13(3):556-563.
16. Bukhari M, Herrick a L, Moore T, Manning J, Jayson MI. Increased nailfold capillary dimensions in primary Raynaud's phenomenon and systemic sclerosis. *Br J Rheumatol.* 1996;35(11):1127-1131. doi:10.1093/rheumatology/35.11.1127
17. Herrick AL. Therapeutic implications from the pathogenesis of Raynaud's phenomenon. *Expert Rev Clin Immunol.* 2017;00(00):1-13. doi:10.1080/1744666X.2017.1279052

18. Sachs K V, Harnke B, Mehler PS, Krantz MJ. Cardiovascular complications of anorexia nervosa: A systematic review. *Int J Eat Disord*. 2016;49(3):238-248. doi:10.1002/eat.22481
19. Pavlova S, Charvat J, Chlumsky J. Flow-mediated vasodilatation in the patients with anorexia nervosa. *Bratisl Lek List*. 2013;114(11):634-636. doi:10.4149/BLL
20. Haller E. Eating disorders. A review and update. *West J Med*. 1992;157(6):658-662.
21. Maricq HR, Carpentier PH, Weinrich MC, et al. Geographic variation in the prevalence of Raynaud's phenomenon: a 5 region comparison. *J Rheumatol*. 1997;24(5):879-889.
22. Lekakis J, Papamichael C, Mavrikakis M, Voutsas A, Stamatelopoulos S. Effect of long-term estrogen therapy on brachial arterial endothelium-dependent vasodilation in women with Raynaud's phenomenon secondary to systemic sclerosis. *AmJCardiol*. 1998;82(0002-9149 LA-eng PT-Clinical Trial PT-Journal Article PT-Randomized Controlled Trial):1555-1557, A8.
23. Nunes Ferreira A, Aldersley R, Efthimiou E. A curious onset of Raynaud's phenomenon after gastric bypass: A case report. *Surg Obes Relat Dis*. 2014;10(6):e109-e111. doi:10.1016/j.soard.2014.01.011
24. Houben AJ, Eringa EC, Jonk AM, Serne EH, Smulders YM, Stehouwer CD. Perivascular Fat and the Microcirculation: Relevance to Insulin Resistance, Diabetes, and Cardiovascular Disease. *Curr Cardiovasc Risk Rep*. 2012;6(1):80-90. doi:10.1007/s12170-011-0214-0
25. Tagami T, Satoh N, Usui T, Yamada K, Shimatsu A, Kuzuya H. Adiponectin in anorexia nervosa and bulimia nervosa. *J Clin Endocrinol Metab*. 2004;89(4):1833-1837. doi:10.1210/jc.2003-031260

Nailfold capillaroscopy is associated with BMI but not with serum levels of adiponectin

4

CHAPTER

The role of oxidative stress in
the development of systemic
sclerosis related vasculopathy

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ABSTRACT

Systemic sclerosis (SSc) is a rare connective tissue disease characterized by autoimmunity, vasculopathy and progressive fibrosis typically affecting multiple organs including the skin. SSc often is a lethal disorder, because effective disease-modifying treatment still remains unavailable. Vasculopathy with endothelial dysfunction, perivascular infiltration of mononuclear cells, vascular wall remodeling and rarefaction of capillaries is the hallmark of the disease. Most patients present with vasospastic attacks of the digital arteries referred to as 'Raynaud's phenomenon', which is often an indication of an underlying widespread vasculopathy. Although autoimmune responses and inflammation are both found to play an important role in the pathogenesis of this vasculopathy, no definite initiating factors have been identified. Recently, several studies have underlined the potential role of oxidative stress in the pathogenesis of SSc vasculopathy thereby proposing a new aspect in the pathogenesis of this disease. For instance, circulating levels of reactive oxygen species (ROS) related markers have been found to correlate with SSc vasculopathy, the formation of fibrosis and the production of autoantibodies. Excess ROS formation is well known to lead to endothelial cell (EC) injury and vascular complications. Collectively, these findings suggest a potential role of ROS in the initiation and progression of SSc vasculopathy. In this review, we present the background of oxidative stress related processes (e.g., EC injury, autoimmunity, inflammation and vascular wall remodeling) that may contribute to SSc vasculopathy. Finally, we describe the use of oxidative stress related read-outs as clinical biomarkers of disease activity and evaluate potential anti-oxidative strategies in SSc.

SYSTEMIC SCLEROSIS

Systemic sclerosis (SSc) is a complex chronic autoimmune disease, characterized by vasculopathy, low-grade chronic inflammation, and fibrosis of the skin and internal organs; the latter causes organ dysfunction, potentially leading to severe morbidity and premature mortality. Vasculopathy is the central feature of the majority of SSc-related complications, for which treatment options are very limited; this has led to a largely unmet medical need. Globally, the incidence rate of SSc appears to have gradually increased over recent decades, with approximately 7-20 million individuals being diagnosed annually.¹ This increase may be due, in part, to improved diagnosis, but it may also be linked to environmental, nutritional, or other lifestyle-related factors that contribute to the surge in cardiometabolic diseases over the same time span. Systemic sclerosis affects women more frequently than men, and previous reports indicate a slightly higher susceptibility to the disease among African Americans as compared to Caucasians.²⁻⁴ The two known subtypes (i.e., limited cutaneous systemic sclerosis and diffuse cutaneous systemic sclerosis) differ in course and prognosis. The diffuse cutaneous subtype is often associated with the manifestation of devastating complications early in the course of the disease, while such complications occur more insidiously in the limited cutaneous subtype. Although auto-immunity, inflammation, and oxidative stress have all been implicated in the pathogenesis of vasculopathy, the definite factors that initiate this disease remain un-identified.⁵

Figure 1: Patients with systemic sclerosis often present with Raynaud's phenomenon (A). Patients may subsequently develop tightening and thickening of the skin of the fingers (sclerodactyly) (B), telangiectasia (C), calcinosis cutis (D) and digital necrosis which may lead to amputation of the digits (E). Written informed consent was obtained from the patients for the publication of these images.

Vasculopathy, which affects both small and large blood vessels, is characterized by endothelial dysfunction, perivascular infiltration of mononuclear cells, extracellular matrix remodeling in the vascular wall, and a loss of capillaries (capillary rarefaction).^{6,7} Raynaud's

phenomenon (RP) (Figure 1), which manifests in the form of recurrent vasospastic attacks that cause episodic discoloration of the extremities in response to cold or emotional stress, is the hallmark of SSc and generally the first detectable sign of the disease.^{5,8,9} Following RP, patients may present with critical ischemia and ulceration, which can result in increased morbidity and mortality, as well as a decreased quality of life.¹⁰ The SSc-specific abnormalities that occur in the capillaries (e.g., dilatation of capillaries in early stages and loss in later phases) can easily and non-invasively be assessed using nailfold capillaroscopy, which is routinely used in the clinical setting.

There is abundant evidence in the literature that elevated production of reactive oxygen species (ROS)— a collective term for oxygen-derived species with enhanced chemical reactivity— or an unfavorable shift in oxidative/reductive tone in favor of a pro-oxidative milieu (a condition known as “oxidative stress”) is involved in a variety of cardiovascular risk factors and diseases (e.g., hypertension, atherosclerosis, and heart failure).^{11–17} However, little is known about the role played by oxidative stress in the development of SSc-related vasculopathy. Following Murrell’s hypothesis¹⁸ that the pathogenesis of SSc is linked to the generation of ROS, several studies have presented evidence of abnormal ROS production in SSc which, if unabated, may subsequently cause damage (Figure 2). Although chronically elevated levels of ROS have been suspected to prolong disease, oxidative stress may also be an initiating factor in the development of SSc vasculopathy. Therefore, a better understanding of the role played by oxidative stress in the development of SSc vasculopathy could help to identify early interventions aimed at delaying the onset of overt symptoms and/or attenuating the course of the disease. In this review, we first describe the physiological role played by ROS in the vascular system. Thereafter, we attempt to explain the role of oxidative stress in the pathogenesis of SSc vasculopathy. Finally, we evaluate the potential of free radical-scavenging therapeutic interventions.

Figure 2: Schematic presentation of the key elements in the pathogenesis of systemic sclerosis. Overproduction of reactive oxygen species may induce endothelial dysfunction and promote a pro-fibrotic and pro-inflammatory state. Moreover, interplay between these processes has been proposed to further cause damage, and various cytokines and chemokines are thought to play an important role in this process.

Abbreviations: AECA: anti-endothelial cell antibodies; CTGF: connective tissue growth factor; EC: endothelial cells; EndoMT: endothelial to mesenchymal transformation; ECM: extracellular matrix; ET-1: endothelin-1; IL: interleukin; MCP: monocyte chemoattractant protein 1; pDC: plasmacytoid dendritic cells; PDGF: platelet derived growth factor; ROS: reactive oxygen species; TGF-β: transforming growth factor beta; Th: T helper cell.

THE ROLE OF ROS IN NORMAL VASCULAR PHYSIOLOGY

PRODUCTION OF REACTIVE OXYGEN SPECIES

Reactive oxygen species were first described by Fenton in 1894;¹⁹ they include free-radical species derived from oxygen such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$) and also non-radical oxidants such as hydrogen peroxide (H_2O_2).^{18,20} Reactive oxygen species can be formed non-enzymatically in reactions catalyzed by metals (as in the Fenton reaction); however, in biology, they are mainly generated through intracellular enzymatic sources, and all vascular cell types (including endothelial cells [ECs], smooth muscle cells, and fibroblasts) are able to produce ROS. The majority of ROS are generated during the production of ATP from molecular oxygen, a process also known as mitochondrial respiration.²¹ During this process, dysregulated mitochondria produce excessive amounts of ROS which can damage all cellular components.²² Mitochondrial

electron transport chains consist of four inner-membrane complexes, with the majority of mitochondrial ROS produced by complexes I and III (Figure 3).²³ Superoxide anion produced by these complexes can be rapidly converted to H_2O_2 by superoxide dismutase family of enzymes, comprised of manganese superoxide dismutase (MnSOD) and copper- and zinc containing superoxide dismutase (Cu,ZnSOD).²⁴ Hydrogen peroxide is then converted to water by glutathione peroxidase, or diffused into the cytosol and, thereby, reduces the damaging effect of ROS to the mitochondria.²⁵ Once ROS is diffused to the cytosol, reactive species are eliminated by the cytosolic antioxidant systems until the cytosolic redox buffer capacity is reached.²⁵ Also, increased mitochondrial peroxynitrite formation may lead to decreased breakdown of ROS, due to the nitration and inactivation of MnSOD.²⁶ Furthermore, cytosolic ROS production by NADPH oxidases may trigger mitochondrial ROS formation through several processes. For instance, the opening of the redox sensitive K_{ATP} channels may lead to changes in the mitochondrial membrane potential, and thereby causing a rise in mitochondrial ROS production.²⁷

Other sources of intracellular ROS production include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) enzymes,²⁸ uncoupled nitric oxide synthases,^{29–33} xanthine oxidases and lipoxygenases.¹⁸ In most of these reactions, ROS formation is a by-product of oxidative metabolism (and is often considered an undesirable result of electron leakage), although this is clearly not the case for NOX enzymes, whose sole purpose is the production of superoxide for signaling purposes or (at much higher rates of production) microbial killing. Mammals are equipped with antioxidant systems that offer protection against the damaging off-target effects of ROS, and several antioxidants and antioxidant enzymes have been described. The antioxidant buffering systems include low-molecular weight substances such as nutritional antioxidants (e.g., vitamin C and vitamin E),³⁴ sulfhydryl (SH)-containing compounds (e.g., thiols such as glutathione),³⁵ and antioxidant enzymes (including superoxide dismutase, catalase, and glutathione peroxidases).³⁶ An imbalance between the production of ROS and their inactivation by antioxidants and reducing enzymes that results in ROS overproduction is referred to as “oxidative stress”.^{37,38} In response to an increase in endogenous production of ROS, an organism may adapt by increasing its antioxidant capacity (commonly referred to as the redox-buffering capacity). If an organism is unable to maintain the appropriate redox homeostasis, disturbances in this balance can cause harmful effects that can ultimately damage the structural and functional components of a cell, leading to the oxidation of proteins, lipids, and DNA.³⁹

Figure 3: A simplified schematic overview of intracellular production of reactive oxygen species (ROS) and the intracellular antioxidant systems. Cytosolic ROS production can be evoked as a response to several stress signals, including hypoxia (**A**). During this process superoxide anion is mainly produced by oxidases, including NADPH oxidase, xanthine oxidase and lipoxygenase. After its production, superoxide is rapidly converted by the antioxidant system to the less damaging radical, hydrogen peroxide and water. Furthermore, cytosolic ROS production can promote mitochondrial ROS production (**B**). Superoxide anion produced by the different complexes of the electron transport chain can easily react with nitric oxide to form peroxy nitrite species. Alternatively, superoxide can also be converted by manganese superoxide dismutase to hydrogen peroxide. Hydrogen peroxide can be either converted to water by glutathione peroxidase, or diffused to the cytosol. If the production of free radicals exceeds the antioxidants buffering capacity, damage to all component of the cell may occur (**C**).

Abbreviations: ATP: adenosine triphosphate; ADP: adenosine diphosphate; FAD/FADH₂: flavin adenine dinucleotide; Fe²⁺: iron; H₂O₂: hydrogen peroxide; H₂O: water; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; iNOS: inducible nitric oxide synthase; NAD⁺/NADH: nicotinamide adenine dinucleotide; NO[•]: nitric oxide; mnSOD: manganese superoxide dismutase; mtNOS: mitochondrial nitric oxide synthase; O₂^{•-}: superoxide; OH[•]: hydroxyl radical; ONOO⁻: peroxynitrite; Cu,ZnSOD: copper-and zinc-containing superoxide dismutase; GP_x: glutathione peroxidase.

REACTIVE OXYGEN SPECIES SIGNALING IN VASCULAR TONE REGULATION

Reactive oxygen species serve as important signaling molecules that regulate a variety of physiological processes in order to maintain appropriate redox homeostasis.⁴⁰ In the vasculature, for example, ROS have been identified as key signaling molecules involved in the fundamental function of vascular smooth muscle cells (VSMC), including the ability of blood vessels to contract or relax.⁴¹ However, the available data underline the complexity of the effects of ROS on the vascular system, as —depending on the conditions— both contraction and relaxation of the vascular muscle may occur.⁴² Superoxide anion has a contractile effect on VSMC, and removal of the endothelium prevents this vasoconstriction, making this process endothelium dependent.⁴³ In line with this finding, the toxicant benzo(a)pyrene has been found to decrease the endothelium-dependent NO-induced vasodilation in retinal arterioles through the production of superoxide.⁴⁴ Vasoconstriction, as an acute response to ROS, is caused by the scavenging of endothelial nitric oxide (NO[•]), another free radical and a potent vasodilator produced in arterial blood vessels to regulate vascular tone and blood flow.⁴⁵ Chronically, vasoconstriction may also be caused by an increased expression of contractile proteins. Likewise, ROS production has previously been associated with a twofold increase in the expression of contractile proteins and microRNA (miR-145), which can up-regulate transcription of contractile protein genes.⁴⁶ In addition, increased contractility of VSMCs by affecting the Ca²⁺ signaling pathway has previously been described.⁴⁷ Several studies have also reported on the vasodilatory effect of ROS, especially in cerebral arteries. Superoxide, generated by xanthine and xanthine oxidase, produces dilation of cerebral arterioles, presumably mediated through potassium channel opening.⁴⁸ Moreover, overproduction of H₂O₂ has been suggested to lead to vasodilatation through an enhanced endothelial response to acetylcholine.^{49–51} A current hypothesis poses that ROS may have a biphasic response, with lower concentrations resulting in vasodilation and higher rates of production causing vasoconstriction,⁴² potentially explaining the contradictory effects of ROS on the vascular system.

NO[•], itself a free-radical reactive nitrogen species (RNS), is also suspected of playing a critical role in vascular homeostasis. NO[•] is a potent activator of soluble guanylyl cyclase, which converts guanosine triphosphate (GTP) to the second messenger cyclic guanosine monophosphate (cGMP), causing vasodilation by increasing calcium handling, which stimulates the contractile function of the VSMC.⁵² NO[•] is synthesized from the amino acid L-arginine by a family of isoenzymes termed nitric oxide synthases (NOS). The synthesis of NO[•] occurs in response to various physicochemical stimuli, including neurotransmitters, shear stress, and growth factors. Furthermore, O₂^{•-} and NO[•] can readily interact with each other to form peroxynitrite (ONOO⁻); this reaction accounts for the vasoconstriction observed by enhanced ROS production and explains the vasorelaxant effects of superoxide dismutase (SOD) in experiments with isolated blood vessels. Prolonged exposure of ECs to peroxynitrite can lead to oxidation of the NOS cofactor, tetrahydrobiopterin, and protein tyrosine nitration, which may impair NOS function following the uncoupling of electron flow from arginine oxidation; these events ultimately

translate into a low NO bioavailability and impaired vasodilatation.^{53,54} Hydrogen sulfide (H_2S) — a reactive signaling molecule belonging to the group of “gasotransmitters”⁵⁵ and a reactive sulfur species (RSS)⁵⁶ produced by ECs and perivascular adipose tissue—may also promote vasodilation through the activation of endothelial NO synthase, the inhibition of cyclic guanosine monophosphate (cGMP) degradation, and by activating potassium channels in VSMCs.⁵⁷ Because of the propensity of ROS, NO, and H_2S to chemically and functionally interact with each other at multiple levels, Cortese-Krott et al. (2017) have underscored the importance of viewing these reactive species as a unified entity (defined as the “reactive species interactome”), rather than as separate signaling entities.⁵⁶

In addition to the direct regulation of vascular tone, ROS are also essential for maintaining O_2 hemostasis by stimulating several transcription factors, such as hypoxia-inducible factor-1 (HIF-1).⁵⁸⁻⁶¹ Hypoxia increases ROS production by a variety of mechanisms, triggering marked alterations in redox signaling.⁶² Furthermore, ROS upregulate vascular endothelial growth factor (VEGF) expression via activation of HIF-1, subsequently leading to angiogenesis.^{63,64} Although these studies shed light on the possible role of ROS in O_2 sensing, their exact role in these and other mechanisms often remains unclear and awaits further clarification. Of note, H_2S is also suspected of being involved in O_2 sensing,⁶⁵ and the availability of both H_2S and ROS is modulated by their interaction with NO,⁵⁶ as mentioned above.

REACTIVE OXYGEN SPECIES SIGNALING IN OTHER CELLULAR PROCESSES AND IMMUNITY

Increasing evidence suggests that ROS also play an important role in a variety of cellular activities through several signaling pathways (e.g., the NF- κ B signaling pathway and the MAPKs signaling pathway).⁶⁶ Several experimental studies have demonstrated that a biphasic effect (i.e., low concentrations increasing the cell number and high concentrations decreasing the cell number) of ROS occur in fibroblasts, cultured smooth muscle cells, cultured ECs, and immortalized lung epithelial cells.⁶⁷⁻⁶⁹ Additionally, human embryonic stem cells have previously been found to engage in spontaneous differentiation when they were cultured under normoxic conditions (21% O_2); however, reduction of the oxygen saturation to 1% O_2 inhibits cell proliferation.⁷⁰ Hypoxia-driven ROS production could partially explain the described observations. Furthermore, both the innate and adaptive immune systems are thought to be affected by ROS production. For example, it has been reported that ROS play a crucial role in T-cell activation, a process that is also associated with an increase in cell ROS production.⁷¹ These findings highlight the importance of ROS in the regulation of several cellular processes.

THE ROLE OF OXIDATIVE STRESS IN SSc VASCULOPATHY

LINK BETWEEN OXIDATIVE AND ENDOTHELIAL CELL DYSFUNCTION/INJURY

The endothelial cell is believed to be a main target for pathological processes in SSc,⁷ and ROS have been implicated as having an important role in this process. In SSc, oxidative stress may cause the activation and damage of ECs,³⁸ leading to EC apoptosis and impairment of cell cell adhesion.⁷² This impairment ultimately increases vascular endothelial permeability, subsequently leading to alterations in EC signal transduction.⁷³ When damaged, vascular ECs produce increased levels of vasoconstrictive mediators (e.g., endothelin-1 [ET-1]) and show an impaired release of NO and prostacyclin.^{74–76} This imbalance in mediators, in addition to several other oxidative and non-oxidative pathways (e.g., the adrenergic mechanism), results in altered vascular tone in favor of vasoconstriction. Consistent with our hypothesis that oxidative stress plays a crucial role in the development of SSc vasculopathy, ECs from SSc patients were previously reported to have the ability to produce H_2O_2 ,⁷⁷ which may inhibit endothelial differentiation due to a reduced octamer-binding transcription factor 4 (Oct-4) expression.⁷⁸ Furthermore, endothelin-1 and angiotensin II (a bioactive peptide of the renin-angiotensin system), which are often elevated in SSc, may upregulate mitochondrial ROS generation through the activation of NADPH oxidase.^{79–81} In addition to this ability to produce ROS, ECs are also more prone to ROS injury due to a deficiency in catalase synthesis.⁸² This deficiency may initiate a self-perpetuating cycle of recurrent RP attacks, increased ROS production (generated during the conversion of xanthine dehydrogenase to xanthine oxidase), more injured ECs, and an inability to defend against oxidative stress.^{83–86} In line with these observations, increased levels of isoprostanes (oxidized lipids and markers of oxidative stress) have been found to correlate with the extent of vascular damage in SSc.^{86,87}

Although inhibition of EC growth has previously been found to be associated with NO overproduction, in SSc, a paradoxical decrease in NO bioavailability has been observed.⁷⁶ This can partially be explained by the rapid reaction of NO[•] with the superoxide anion $O_2^{\cdot-}$, forming peroxynitrite.⁸⁸ With disease progression, the production of eNOS is further downregulated.⁸⁹ As a result of EC injury and subsequent vascular remodeling, SSc patients show specific capillaroscopic patterns consisting of micro-hemorrhages, as well as dilated, giant, and malformed capillaries.⁹⁰ These nailfold patterns are positively associated with the degree of vasospasm and ischemia, further indicating that ROS plays a role in the occurrence of microvascular injury.⁹¹

LINK BETWEEN OXIDATIVE STRESS AND AUTO-IMMUNITY

Several SSc-specific auto-antibodies (e.g., anti-topoisomerase 1, anti-centromere antibodies, anti-RNA polymerase III, anti-U3-RNP, TH/To RNP, U1-RNP, and PM-Scl) have previously been identified, and ROS are thought to be involved in the process leading to autoimmunity. More recently, subcutaneous injections of agents generating OH[•] or HOCl (another ROS) in mice induced cutaneous and lung fibrosis, as well as the production

of serum anti-DNA topoisomerase 1 antibodies.⁸⁴ Furthermore, it has been shown that injections of ONOO⁻ generating agents induce the production of anti-centromere B antibodies in the absence of serum anti-DNA topoisomerase 1 antibodies. These findings support the hypothesis that bursts of ROS may cause the oxidation of DNA-topoisomerase-1, which may lead to immunological intolerance to this nuclear antigen. In line with these findings, mice exposed to HOCl developed anti-DNA topoisomerase I antibodies and diffuse cutaneous SSc with pulmonary fibrosis.⁹²

Autoimmunity may occur through the impairment of DNA repair mechanisms (Figure 2).⁹³ For example, it has previously been established that 8-oxo-7 hydrodeoxyguanosine levels in lymphocytes were increased in several rheumatic diseases, as compared to healthy controls.⁹⁴ More recently, autoantibodies against methionine sulfoxide reductase A, one of the antioxidant repair enzymes, have been detected in 33% of SSc patients.⁸⁷ This finding supports the hypothesis that an altered defense mechanism may contribute to the development of vasculopathy. In addition, this finding is also indicative of a promutagenic DNA lesion, presumably induced by ROS and defective DNA repair mechanisms.⁹⁴

Recently, several studies have investigated the role of a new class of antibodies that may be directly linked to the pathological processes that occur even in the early stages of SSc. For instance, anti-endothelial cell antibodies (AECAs) are thought to play an important role in EC injury promoting the development of vasculopathy.⁹⁵⁻⁹⁸ Anti-endothelial cell antibodies may induce endothelial perturbation, an increase in adhesion molecule expression (ICAM-1, VCAM-1, and E-selectin) and stimulate the secretion of pro-inflammatory cytokines and chemokines.⁹⁹ The formation of AECAs is thought to be initiated by the exposure of the nuclear contents of damaged ECs,¹⁰⁰ which may very well be induced by excessive ROS production. Moreover, some evidence provided using a molecular cloning strategy suggests that these antibodies (e.g., AECAs) cause oxidative imbalance and subsequently lead to endothelial apoptosis, suggesting causal involvement in the disease process.¹⁰¹ Some evidence supports the hypothesis that heat shock proteins (HSP) play an important role in the pathogenesis of SSc.^{102,103} Heat shock proteins are a family of immunogenic proteins that are found in all organism cells, and are essential for various cellular function. These proteins have chaperon properties and facilitate in protein folding, assembly, and intracellular transport.¹⁰⁴ The synthesis of HSP is increased in response to a variety of stressful stimuli, such as hyperthermia, hypoxia, inflammation and auto-immunity.^{105,106} Heat shock proteins have previously been found to stimulate antigen presenting cells, which then activate the adaptive immune cells, suggesting a critical role of HSPs in the immune system.¹⁰⁴ Furthermore, increased levels of antibodies to HSP have previously been demonstrated in several immune diseases, including atherosclerosis, type 1 diabetes mellitus and various auto-immune rheumatic diseases.^{107,108} Moreover, the 60-kd heat shock protein (HSP60) was previously found to be a target antigen for AECAs, and thereby induce apoptosis of ECs in variety of rheumatic diseases, including SSc. In support, serum HSP70 levels are also found to be increased in SSc patients, and are associated with the occurrence of fibrosis, oxidative stress and inflammation.¹⁰³ Moreover,

expression of HSP47 was found to be linked to the overproduction of type I collagen in cultured SSc fibroblast.¹⁰⁹ These results shed light on the potential of these markers as serological biomarkers of cellular stress. Conversely, there is some evidence supporting the postulation that HSPs also have a beneficial effect following oxidation.¹¹⁰ These effects are likely linked to the sensor ability of redox changes and cytoprotective effects of HSPs. However, the exact role in the pathogenesis of SSc has to be elucidated further.

Age related auto-immunity is also thought to play an important role in SSc. Approximately 21% of all SSc patients are aged 65 years or older at diagnosis.¹¹¹ This observation has instigated great interest in the role of key determinants of biological ageing (e.g., telomere attrition, senescent cells) in the pathogenesis of SSc. Autoimmunity in the elderly population is also believed to be promoted by accelerated cellular senescence,¹¹² and through the increased affinity of T-cells to self-antigens.¹¹³ It was previously demonstrated that the age of disease onset strongly influences clinical signs of SSc, the presence organ involvement and outcome. Moreover, the elderly SSc patients, compared to their younger counterparts, were more frequently diagnosed with cardiopulmonary organ involvement.¹¹⁴ A study conducted by Shiels et al. (2011) demonstrated that patients diagnosed with dcSSc exhibit features consistent with accelerated biological ageing, and this was found to be independent of increasing age.¹¹⁵ This study concluded that abnormal telomere biology may induce the development clinical signs in SSc patients. Supporting this hypothesis, a previously conducted study found that the telomere length of SSc patients, and their family members, were on average 3 Kb shorter than age-matched controls. In addition, they stated that the telomeric length in SSc patients did not decrease significantly with age.¹¹⁶ This reduction in telomere length can partially be explained by the elevated oxidative stress levels in these patients, which may accelerate telomere erosion.¹¹⁷ Moreover, this observation may also be explained by the presence of autoantibodies against telomere binding proteins.

LINK BETWEEN OXIDATIVE STRESS AND INFLAMMATION

Chronic ROS overproduction may stimulate a pro-inflammatory state in SSc.^{118,119} The early phase of SSc is characterized by perivascular mononuclear cell infiltration⁷ and the activation of the innate and adaptive immune system, which results in B-cell and T-cell activation.¹²⁰ These cells secrete pro-inflammatory cytokines (including IL-1, IL-6 and IL-8) and chemokines that further stimulate ROS production.^{18,121} However, ROS have been reported to promote activation of a variety of inflammation-associated transcription factors (e.g., nuclear factor-kappa B [NF- κ B], activator protein-1 [AP-1], p53, signal transducer and activator of transcriptions 3 [STAT3], HIF-1 α , and NF-E2 related factor-2 [Nrf2]). This process can further aggravate the inflammatory response through the production of a variety of inflammatory mediators.¹²² Although these findings emphasize that the development of SSc vasculopathy is due to interconnected pathways of both inflammatory processes, as well as oxidative stress, the level of oxidative stress in SSc seems to exceed that which can be explained solely by the inflammatory process. Therefore, our hypothesis that oxidative stress initiates the development of vasculopathy appears to be reasonable.

LINK BETWEEN OXIDATIVE STRESS AND VASCULAR WALL REMODELING

Vascular remodeling affects a variety of sites, including the lungs, the heart, the skin and the kidneys, resulting in critical luminal narrowing of the vessel (Figure 4 and Figure 5). Severe complications, such as pulmonary arterial hypertension (PAH) and scleroderma renal crisis (SRC), are accompanied by this luminal narrowing. The occlusion of the microvasculature due to intimal and media proliferation may result in an impaired tissue supply of oxygen and nutrients, leading to increased ROS production and, eventually, to a loss of vasculature.⁶ In addition, several signals involved in SSc pathogenesis, including transforming growth factor-beta (TGF- β), PDGF, and ET-1 modulate the expression of NOX, which may lead to the formation of fibrosis by up-regulating ROS production.¹²³

Figure 4: Simplified representation of vascular wall remodeling, leading to critical luminal narrowing of a small vessel as can be seen in patients with systemic sclerosis. The early events in the pathogenesis of systemic sclerosis are characterized by endothelial cell (EC) injury, and the lightning bolt represents the initiating factors causing this injury. Reactive oxygen species may play an important initiating role in this process. Following endothelial cell injury, endothelial cell apoptosis and loss of integrity of the endothelial lining may consequently occur. With the progression of the disease, basement membrane (**continuous grey bold line**) thickening and disruption of the internal elastic lamella are also observed (**dashed line**). Concomitantly, peripheral-blood mononuclear cells (**round nuclear cells**) and vascular smooth muscle cells (**pink oval-shaped cells**) infiltrate the intimal layer. This process initiates the formation of intimal fibrosis through differentiation of the vascular smooth muscle cells into myofibroblasts, which produce an increased amount of extracellular matrix proteins. Alongside this process, adventitial fibrosis is formed due to increased production of densely packed collagen bundles (**pink bold lines**).

Figure 5: Histopathological alterations of a dermal vessel. Patient, a 62-year-old female, was referred to the dermatologist due to a rash of the lower limbs. All images were taken at 40x magnification, **Figure A** shows Haematoxylin and eosin stained tissue, **Figure B** shows Verhoeff stained tissue, **Figure C** shows alpha-smooth muscle actin stained tissue and **Figure D** shows ERG stained tissue. The vessel displayed above shows prominent fibroproliferative alterations, leading to intima and media proliferation; subsequently causing severe narrowing of the vessel lumen and thickening of vessel walls (**Figure A**). Also, disruption of the internal elastic lamella can be observed (**Figure B**), with media cells invading the intimal layer (**Figure C**). This process is accompanied by slight perivascular infiltration of inflammatory cells.

Reactive oxygen species have been implicated in several SSc-related processes, including the stimulation of fibroblast proliferation, collagen-gene expression, and phenotype conversion of myofibroblast.⁸⁸ An imbalance between collagen and elastin and the infiltration of the vessel wall by VSMCs, mononuclear cells, and other inflammatory cells, as well as an increased production of cytokines, MMP, chemokines (MCP-1), cell adhesion molecules, and growth factors (TGF- β , *CTGF*, *PDGF*), all contribute to vascular wall thickening. Accordingly, it has been observed that extracellular matrix deposition into the vessel wall results from disproportionate fibroblast activity with increased serum levels of hyaluronan, matrix metalloproteinases (MMP), and tissue inhibitors of metalloproteinases (TIMPs).¹²⁴ TGF- β , in particular, promotes the synthesis of collagen, glycosaminoglycans, and fibronectin by fibroblast, and reduces the collagenase-synthesizing capacity of fibroblasts.¹²⁵

Perivascular cells including, but not limited to, smooth muscle cells (SMC), and in particular VSMCs, are thought to play a key role in maintaining vascular integrity. During physiological processes, these cells have a contractile function, and thereby regulate blood flow. Current literature suggest that intimal hyperplasia may be formed by migration and proliferation of medial SMC and adventitial fibroblast,⁶ as a response to various stimuli including oxidative stress. Subsequently, VSMCs produce excessive amount of ECM to form a fibrotic vascular lesion. Moreover, following the initial vascular

injury, endothelial progenitor cells are mobilized from the bone marrow, recruited to the site of the vascular lesion and differentiate into a variety of cells, including VSMCs.¹²⁶ Proliferation of VSMCs is usually regulated by NO, which inhibits cell proliferation through upregulation of cGMP. However, due to the impaired NO production in SSc, processes related to the inhibition of cell proliferation may be altered, and ECM production may be promoted further. In addition to the decreased production of NO, cell proliferation is also promoted by upregulation of ET_B receptor on SMCs.¹²⁷ In addition, migration and proliferation of VSMCs is also likely due to the impaired interaction between ECs and VSMCs. This impaired interaction, which is characterized by a decreased expression of α -SMA in VSMCs, is thought to induce phenotypic modulation in VSMC. This phenotypic modulation induces VSMCs to act in a more migratory and proliferative manner.¹²⁸

Vascular remodeling is also promoted by the ability of ECs to convert into myofibroblast under hypoxic and pro-oxidative milieu, a process known as “endothelial to mesenchymal transition”.¹²⁹ On the other hand, NO has been reported to have an antifibrotic effect in experimental animal models.¹³⁰ NO can directly activate several transcription factors (such as nuclear factor- κ B, specificity protein-1, and activator protein) to inhibit collagen gene expression.¹³¹ In addition, NO mediates prolyl hydroxylase expression, which is important in the posttranslational processing of collagen.¹³² Therefore, the formation of fibrosis may be stimulated by the decreased bioavailability of NO in SSc patients.

HIF-1 α is generally increased substantially as a response to low oxygen concentrations, which generally promotes ROS production. This upregulation of HIF-1 controls the expression of several genes involved in processes such as erythropoiesis, angiogenesis, glucose metabolism, cell proliferation, and cell apoptosis.^{133,134} Tissue hypoxia usually triggers angiogenesis in order to improve oxygenation of hypoxic tissue. As a response to chronic hypoxia due to progressive loss of vasculature, levels of vascular endothelial growth factor (VEGF) often increase. Accordingly, both receptors for VEGF (e.g., VEGF receptors 1 and 2) have previously been found to be overexpressed in the skin and serum of SSc patients.¹³⁵ Reactive oxygen species have been implicated to stimulate the induction of VEGF expression in variety of cells, including ECs, VSMC, and macrophages.^{136–138} Simultaneously, VEGF further increases the production of ROS through the activation of NADPH oxidase in ECs.¹³⁹

BIOMARKERS OF OXIDATIVE STRESS

In the past decade, several *in vitro* and *in vivo* studies have demonstrated the role played by the overproduction of free radicals in the pathophysiology of SSc, and several biomarkers of this process have been described. These biomarkers include oxidized low-density lipoprotein, malondialdehyde (MDA), isoprostanes, and circulating total free thiols. Free thiol groups (i.e., -SH) can be oxidized by ROS and other reactive species and are active components of the antioxidant buffer capacity, indicating that their extracellular level can be interpreted as a direct reflection of the overall redox status.^{35,56,140} In 1992, Lau and colleagues were the first to observe that plasma thiol concentration was reduced in patients with SSc.¹⁴¹ More recently, we have demonstrated that patients with SSc show

decreased levels of free thiols, indicating high oxidative stress. Interestingly, levels of free thiols clearly increased following a cooling experiment of the lower forearm, simulating a Raynaud's attack.¹⁴² Following their initial observation, Lau et al. (1992) also reported increased levels of MDA, which is a measure of lipid peroxidation.¹⁴³ Additionally, increased urinary levels of 8-isoprostanes were previously found in SSc patients in the early stages of the disease.¹⁴⁴ It has also been reported that 8-isoprostane in exhaled breath condensate of SSc patients is increased.¹⁴⁵ Similarly, it was demonstrated that bronchoalveolar lavage fluid from SSc patients with fibrosing alveolitis contained increased levels of 8-isoprostanes.¹⁴⁶ Furthermore, another study showed that the level of 8-isoprostane not only correlates with the severity of pulmonary fibrosis, but also with the extent of renal vascular damage, and immunological abnormalities in SSc.⁸⁶ Collectively, these studies have generated increasing interest in the hypothesis that oxidative stress may play a significant role in the pathogenesis of SSc, and subsequently promote vascular injury. However, in order to optimally use these biomarkers, larger studies investigating the value of these markers as predictors of disease (and disease progression) should be performed.

INTERVENTIONAL STRATEGIES

Clear evidence indicates the crucial role played by oxidative stress in the occurrence of SSc vasculopathy. For instance, it has been hypothesized that in SSc, ECs are unable to endure prolonged oxidative stress, either through a lack of antioxidant defense mechanisms or because these antioxidant mechanisms are compromised.⁸³ As extensively described elsewhere,^{38,83} several studies have investigated the effect of antioxidants in SSc patients. These studies all shared the postulation that antioxidant supplements would decrease susceptibility to oxidative stress-induced tissue damage. Despite some promising results, larger trials have been disappointing.^{83,147} For instance, Niwa et al (1985) reported that three out of three SSc patients benefitted from liposomal-encapsulated superoxide dismutase injections.¹⁴⁸ However, Correa et al. (2014) demonstrated that, when taken orally (1800mg/day), free radical scavenger N-acetyl-L-cysteine (NAC) showed no vasodilator effect on the microcirculation of the hands after four weeks of treatment in SSc patients.¹⁴⁹ Conversely, others have reported on the beneficial effect of NAC in SSc patients. For example, NAC has been found to diminish cellular ROS in fibroblast and replenish free cellular thiols.^{77,88,150} In addition, an *in vitro* study, conducted in SSc patients, showed that NAC inhibits fibroblast proliferation and collagen synthesis and reduces the formation of peroxynitrite (ONOO⁻) by activated lung macrophages.¹⁵¹ Moreover, Rosata et al. (2009) demonstrated that treatment with NAC led to a reduction in the number of digital ulcers and decreased the number of RP attacks.¹⁵²

Denton et al. (1999) conducted a randomized parallel group study in 40 RP patients (20 SSc patients, 15 primary RP patients and five secondary RP patients).¹⁵³ These patients were randomized to receive either 500 mg daily of the lipid-lowering antioxidant probucol or 20 mg daily of nifedipine. Those patients treated with probucol were found to have a significant reduction in the frequency and severity of Raynaud's attacks, as well as a rise in LDL oxidation lag time. In addition, hydrogen sulfide (H₂S), known for its strong

antioxidant and vasodilator capacity, was demonstrated to have beneficial effects on bleomycin-induced pulmonary fibrosis in a mice model.¹⁵⁴ Moreover, Wang et al. (2016) demonstrated that H₂S could improve SSc-related organ fibrosis through the inhibition of the inflammatory reaction and the reduction of TGF- β 1 expression.¹⁵⁵ These data place H₂S in the spotlight as an effective therapeutic agent in SSc patients with organ involvement. Furthermore, there is some evidence indicating that soluble guanylate cyclase (sGC) may be beneficial in SSc. For instance, large randomized controlled clinical trials conducted with riociguat, which directly stimulates sGC in an NO-independent manner, have shown an increase in mean 6-minute walking distance and an improvement of pulmonary vascular resistance and cardiac index.¹⁵⁶

Regarding more conventional therapeutic strategies in SSc, the exploratory study conducted by Erre and colleagues suggested that a standard course of iloprost therapy, a potent vasodilator, may reduce oxidative stress in SSc patients.¹⁵⁷ This effect appeared to be more consistent in patients in the early phase of the disease. Furthermore, an experimental study conducted by Rafikova et al. (2013) showed that the ET receptor antagonist bosentan (250 mg/kg/day; day 10–21), which is generally prescribed to reduce the number of new DU, not only prevented an increase in right ventricle peak systolic pressure and right ventricle hypertrophy, but also reduced oxidative stress and protein nitration.¹⁵⁸

FUTURE PERSPECTIVES

Following Murrell's hypothesis,¹⁸ several studies have investigated the role played by oxidative stress in the pathogenesis of SSc vasculopathy. These studies have not only substantially improved our understanding of this complex disease, but several findings, as presented in this review, support the hypothesis that oxidative stress contributes to, and may even initiate, the occurrence of vasculopathy. This contrasts with the finding that most therapeutic intervention studies with antioxidants have been disappointing. However, some have proposed that these non-conventional therapeutic strategies may only be beneficial in the early stages of the disease, when less severe damage and perhaps lower levels of ROS occur. However, this acts as a double-edged sword. Although universally recognized diagnostic criteria for very early diagnosis of the disease (VEDOSS criteria) have been introduced, these criteria include patients who already have developed EC damage, vasculopathy, and specific antibodies. The inability to identify patients with Raynaud's who are at increased risk of developing SSc is the primary challenge. Any therapy initiated later in the course of the disease may only mitigate disease processes and symptoms, without actually modifying the disease course and outcome. We postulate that interventions administered early in the course of the disease, before the occurrence of irreversible fibrosis, may prevent irreversible changes by altering the damaging properties of ROS and thereby changing the disease's course. Although we are encouraging new trials to investigate the effect of antioxidants on SSc, which should include patients in the early stages of the disease, we emphasize that new trials may only be successful when the underlying pathology is fully clarified.

REFERENCES

1. Nikpour M, Stevens WM, Herrick AL, Proudman SM. Epidemiology of systemic sclerosis. *Best Pract Res Clin Rheumatol*. 2010;24(6):857-869. doi:10.1016/j.berh.2010.10.007.
2. Laing TJ, Gillespie BW, Toth MB, et al. Racial differences in scleroderma among women in Michigan. *Arthritis Rheum*. 1997;40(4):734-742. doi:10.1002/art.1780400421.
3. Ranque B, Mouthon L. Geoepidemiology of systemic sclerosis. *Autoimmun Rev*. 2010;9(5):A311-A318. doi:10.1016/j.autrev.2009.11.003.
4. Mayes MD, Lacey JV., Beebe-Dimmer J, et al. Prevalence, incidence, survival, and disease characteristics of systemic sclerosis in a large US population. *Arthritis Rheum*. 2003;48(8):2246-2255. doi:10.1002/art.11073.
5. Gabrielli A, Avvedimento EV., Krieg T. Scleroderma. *N Engl J Med*. 2009;360(19):1989-2003. doi:10.1056/NEJMra0806188.
6. Fleming JN, Nash RA, Mahoney WM, Schwartz SM. Is scleroderma a vasculopathy? *Curr Rheumatol Rep*. 2009;11(2):103-110. doi:10.1007/s11926-009-0015-3.
7. Mostmans Y, Cutolo M, Giddelo C, et al. The role of endothelial cells in the vasculopathy of systemic sclerosis: A systematic review. *Autoimmun Rev*. 2017. doi:10.1016/j.autrev.2017.05.024.
8. Herrick AL. Pathogenesis of Raynaud's phenomenon. *Reumatologia*. 2005;13(3):62-65. doi:10.1093/rheumatology/keh552.
9. LeRoy EC, Medsger J. Criteria for the classification of early systemic sclerosis. *J Rheumatol*. 2001;28(7):1573-1576.
10. Almeida C, Almeida I, Vasconcelos C. Quality of life in systemic sclerosis. *Autoimmun Rev*. 2015;14(12):1087-1096. doi:10.1016/j.autrev.2015.07.012.
11. Tocchetti CG, Stanley BA, Murray CI, et al. Playing with Cardiac "Redox Switches": The "HNO Way" to Modulate Cardiac Function. *Antioxid Redox Signal*. 2011;14(9):1687-1698. doi:10.1089/ars.2010.3859.
12. Wu J, Xia S, Kalionis B, Wan W, Sun T. The Role of Oxidative Stress and Inflammation in Cardiovascular Aging. *Biomed Res Int*. 2014;2014. doi:10.1155/2014/615312.
13. Barry-Lane PA, Patterson C, Van Der Merwe M, et al. p47phox is required for atherosclerotic lesion progression in ApoE^{-/-} mice. *J Clin Invest*. 2001;108(10):1513-1522. doi:10.1172/JCI200111927.
14. Heinloth A, Heermeier K, Raff U, Wanner C, Galle J. Stimulation of NADPH oxidase by oxidized low-density lipoprotein induces proliferation of human vascular endothelial cells. *J Am Soc Nephrol*. 2000;11(10):1819-1825.
15. Hirotsu S, Otsu K, Nishida K, et al. Involvement of nuclear factor-kappaB and apoptosis signal-regulating kinase 1 in G-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy. *Circulation*. 2002;105(4):509-515.
16. Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA, Harrison DG. Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. *Circulation*. 1997;95(3):588-593. doi:10.1161/01.cir.95.3.588.
17. Nakamura K, Fushimi K, Kouchi H, et al. Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-alpha and angiotensin II. *Circulation*. 1998;98(8):794-799. doi:10.1161/01.CIR.98.8.794.
18. Murrell DF. A radical proposal for the pathogenesis of scleroderma. *J Am Acad Dermatol*. 1993;28(1):78-85. doi:10.1016/0190-9622(93)70014-K.
19. Fenton HJH. Oxidation of tartaric acid in presence of iron. *J Chem Soc*. 1894;65:899-910. doi:10.1039/ct8946500899.

20. Cumpstey A, Feelisch M. Free radicals in inflammation: from molecular and cellular mechanisms to the clinic. In: Cavaillon, Jean-Marc and Singer M, ed. *Inflammation: From Molecular and Cellular Mechanisms to the Clinic*. 1st ed. Wiley-Blackwell; 2017:695-726.
21. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell*. 2005;120(4):483-495. doi:10.1016/j.cell.2005.02.001.
22. Bolisetty S, Jaimes EA. Mitochondria and reactive oxygen species: Physiology and pathophysiology. *Int J Mol Sci*. 2013;14(3):6306-6344. doi:10.3390/ijms14036306.
23. Berg JM, Tymoczko JL, Stryer L. The Respiratory Chain Consists of Four Complexes: Three Proton Pumps and a Physical Link to the Citric Acid Cycle. In: *Biochemistry*. Vol New York. ; 2002:320-323.
24. Miriyala S, Spasojevic I, Tovmasyan A, et al. Manganese superoxide dismutase, MnSOD and its mimics. *Biochim Biophys Acta - Mol Basis Dis*. 2012;1822(5):794-814. doi:10.1016/j.bbadis.2011.12.002.
25. Maharjan S, Oku M, Tsuda M, Hoseki J, Sakai Y. Mitochondrial impairment triggers cytosolic oxidative stress and cell death following proteasome inhibition. *Sci Rep*. 2014;4:1-11. doi:10.1038/srep05896.
26. Daiber A. Redox signaling (cross-talk) from and to mitochondria involves mitochondrial pores and reactive oxygen species. *Biochim Biophys Acta - Bioenerg*. 2010;1797(6-7):897-906. doi:10.1016/j.bba-bio.2010.01.032.
27. Garlid KD, Dos Santos P, Xie ZJ, Costa ADT, Paucek P. Mitochondrial potassium transport: The role of the mitochondrial ATP-sensitive K⁺ channel in cardiac function and cardioprotection. *Biochim Biophys Acta - Bioenerg*. 2003;1606(1-3):1-21. doi:10.1016/S0005-2728(03)00109-9.
28. Bedard K, Krause K-H. The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology. *Physiol Rev*. 2007;87(1):245-313. doi:10.1152/physrev.00044.2005.
29. Taniyama Y, Griendling KK. Reactive Oxygen Species in the Vasculature: Molecular and Cellular Mechanisms. *Hypertension*. 2003;42(6):1075-1081. doi:10.1161/01.HYP.0000100443.09293.4F.
30. Nishino T, Okamoto K, Eger BT, Pai EF, Nishino T. Mammalian xanthine oxidoreductase - Mechanism of transition from xanthine dehydrogenase to xanthine oxidase. *FEBS J*. 2008;275(13):3278-3289. doi:10.1111/j.1742-4658.2008.06489.x.
31. Kawashima EP, Channon KM, Adlam D, et al. Relationships between nitric oxide-mediated endothelial function, eNOS coupling and blood pressure revealed by eNOS-GTP cyclohydrolase 1 double transgenic mice. *Exp Physiol*. 2007;92(92):119-126. doi:10.1113/expphysiol.2006.035113.
32. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol*. 2003;552(2):335-344. doi:10.1111/j.1469-7793.2003.00335.x.
33. Landmesser U, Dikalov S, Price SR, et al. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest*. 2003;111(8):1201-1209. doi:10.1172/JCI200314172.
34. Benfeito S, Oliveira C, Soares P, et al. Antioxidant therapy: Still in search of the "magic bullet." *Mitochondrion*. 2013;13(5):427-435. doi:10.1016/j.mito.2012.12.002.
35. Turell L, Radi R, Alvarez B. The thiol pool in human plasma: The central contribution of albumin to redox processes. *Free Radic Biol Med*. 2013;65:244-253. doi:10.1016/j.freeradbiomed.2013.05.050.
36. Matés JM, Pérez-Gómez C, Núñez de Castro I. Antioxidant enzymes and human diseases. *Clin Biochem*. 1999;32(8):595-603. doi:10.1016/S0009-9120(99)00075-2.
37. Betteridge DJ. What is oxidative stress? *Metabolism*. 2000;49(2 Suppl 1):3-8. doi:10.1016/S0026-0495(00)80077-3.
38. Grygiel-Górniak B, Puszczewicz M. Oxidative Damage and Antioxidative Therapy in Systemic Sclerosis. *Mediators Inflamm*. 2014;1-11. doi:10.1155/2014/389582.
39. Harman D. Aging: A Theory Based on Free Radical and Radiation Chemistry. *J Gerontol*. 1956;11(3):298-300. doi:10.1093/geronj/11.3.298.

40. Jones DP, Sies H. The Redox Code. *Antioxid Redox Signal*. 2015;23(9):734-746. doi:10.1089/ars.2015.6247.
41. MacKay CE, Knock GA. Control of vascular smooth muscle function by Src-family kinases and reactive oxygen species in health and disease. *J Physiol*. 2015;593(17):3815-3828. doi:10.1113/jphysiol.2014.285304.
42. Faraci FM. Reactive oxygen species: influence on cerebral vascular tone. *J Appl Physiol*. 2006;100(8750-7587 (Print)):739-743. doi:10.1152/japplphysiol.01044.2005.
43. Katusic Z, Vanhoutte P. Superoxide anion is an endothelium-derived contracting factor. *Am J Physiol*. 1989;257(1 Pt 2):H33-7.
44. Kamiya T, Nagaoka T, Omae T, Ono S, Otani S, Yoshida A. Benzo(e)Pyrene, a toxic element in cigarette smoke, inhibits endothelium-dependent nitric oxide-mediated dilation of porcine retinal arterioles via enhanced superoxide production. *Investig Ophthalmol Vis Sci*. 2014;55(13):4349.
45. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*. 1991;43(2):109-142. doi:10.1017/CBO9781107415324.004.
46. Chettimada S, Ata H, Rawat DK, et al. Contractile protein expression is upregulated by reactive oxygen species in aorta of Goto-Kakizaki rat. *Am J Physiol Heart Circ Physiol*. 2014;306(2):H214-24. doi:10.1152/ajpheart.00310.2013.
47. Touyz RM, Schiffrin EL. Reactive oxygen species in vascular biology: Implications in hypertension. *Histochem Cell Biol*. 2004;122(4):339-352. doi:10.1007/s00418-004-0696-7.
48. Wei EP, Kontos HA, Beckman JS. Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide, and peroxynitrite. *Am J Physiol*. 1996;271(3 Pt 2):H1262-H1266.
49. Zhou Y, Yan H, Guo M, Zhu J, Xiao Q, Zhang L. Reactive oxygen species in vascular formation and development. *Oxid Med Cell Longev*. 2013. doi:10.1155/2013/374963.
50. Thomas SR, Chen K, Keaney JF. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. *J Biol Chem*. 2002;277(8):6017-6024. doi:10.1074/jbc.M109107200.
51. Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circ Res*. 2000;86(3):347-354. doi:10.1161/01.RES.86.3.347.
52. Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci*. 1977;74(8):3203-3207. doi:10.1073/pnas.74.8.3203.
53. Dimmeler S, Zeiher a M. Reactive oxygen species and vascular cell apoptosis in response to angiotensin II and pro-atherosclerotic factors. *Regul Pept*. 2000;90(1-3):19-25. doi:S0167011500001051 [pii].
54. Aliev G, Bodin P, Burnstock G. Free radical generators cause changes in endothelial and inducible nitric oxide synthases and endothelin-1 immunoreactivity in endothelial cells from hyperlipidemic rabbits. *Mol Genet Metab*. 1998;63(3):191-197. doi:10.1006/mgme.1997.2664.
55. Wang R. Two's company, three's a crowd: can H₂S be the third endogenous gaseous transmitter? *FASEB J*. 2002;16(13):1792-1798. doi:10.1096/fj.02-0211hyp.
56. Cortese-Krott MM, Koning A, Kuhnle GGC, et al. The Reactive Species Interactome: Evolutionary Emergence, Biological Significance, and Opportunities for Redox Metabolomics and Personalized Medicine. *Antioxid Redox Signal*. 2017:684-712. doi:10.1089/ars.2017.7083.
57. Bltowski J, Jamroz-Wisniewska A. Hydrogen sulfide and endothelium-dependent vasorelaxation. *Molecules*. 2014;19(12):21506-21528. doi:10.3390/molecules191221183.
58. Brocato J, Chervona Y, Costa M. Molecular responses to hypoxia-inducible factor 1 and beyond. *Mol Pharmacol*. 2014;85(5):651-657. doi:10.1124/mol.113.089623.

59. Friedman JK, Nitta CH, Henderson KM, et al. Intermittent hypoxia-induced increases in reactive oxygen species activate NFATc3 increasing endothelin-1 vasoconstrictor reactivity. *Vascul Pharmacol*. 2014;60(1):17-24. doi:10.1016/j.vph.2013.11.001.
60. Chandel NS, McClintock DS, Feliciano CE, et al. Reactive oxygen species generated at mitochondrial Complex III stabilize hypoxia-inducible factor-1 α during hypoxia: A mechanism of O₂sensing. *J Biol Chem*. 2000;275(33):25130-25138. doi:10.1074/jbc.M001914200.
61. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc Natl Acad Sci U S A*. 1998;95(20):11715-11720. doi:10.1073/pnas.95.20.11715.
62. Smith KA, Waypa GB, Schumacker PT. Redox signaling during hypoxia in mammalian cells. *Redox Biol*. 2017;13:228-234. doi:10.1016/j.redox.2017.05.020.
63. Arbiser JL, Petros J, Klawter R, et al. Reactive oxygen generated by Nox1 triggers the angiogenic switch. *Proc Natl Acad Sci U S A*. 2002;99(2):715-720. doi:10.1073/pnas.022630199.
64. Xia C, Meng Q, Liu L-Z, Rojanasakul Y, Wang X-R, Jiang B-H. Reactive oxygen species regulate angiogenesis and tumor growth through vascular endothelial growth factor. *Cancer Res*. 2007;67(22):10823-10830. doi:10.1158/0008-5472.CAN-07-0783.
65. Olson KR. Hydrogen Sulfide as an Oxygen Sensor. *Antioxid Redox Signal*. 2015;22(5):377-397. doi:10.1089/ars.2014.5930.
66. Zhang J, Wang X, Vikash V, et al. ROS and ROS-Mediated Cellular Signaling. *Oxid Med Cell Longev*. 2016;2016. doi:10.1155/2016/4350965.
67. Arnold RS, Shi J, Murad E, et al. Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. *Proc Natl Acad Sci U S A*. 2001;98(10):5550-5555. doi:10.1073/pnas.101505898.
68. Day RM, Suzuki YJ. Cell proliferation, reactive oxygen and cellular glutathione. *Dose Response*. 2005;3(3):425-442. doi:10.2203/dose-response.003.03.010.
69. Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radic Biol Med*. 1995;18(4):775-794. doi:10.1016/0891-5849(94)00198-S.
70. Ezashi T, Das P, Roberts RM. Low O₂ tensions and the prevention of differentiation of HES cells. *Proc Natl Acad Sci*. 2005;102(13):4783-4788. doi:10.1073/pnas.0501283102.
71. Devadas S, Zaritskaya L, Rhee SG, Oberley L, Williams MS. Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and fas ligand expression. *J Exp Med*. 2002;195(1):59-70. doi:10.1084/jem.20010659.
72. Sgonc R, Gruschwitz MS, Dietrich H, Recheis H, Gershwin ME, Wick G. Endothelial cell apoptosis is a primary pathogenetic event underlying skin lesions in avian and human scleroderma. *J Clin Invest*. 1996;98(3):785-792. doi:10.1172/JCI118851.
73. Fleischmajer R, Perlish JS. Capillary alterations in scleroderma. *J Am Acad Dermatol*. 1980;2(2):161-170. doi:10.1016/S0190-9622(80)80396-3.
74. Freedman RR, Girgis R, Mayes MD. Endothelial and adrenergic dysfunction in Raynaud's phenomenon and scleroderma. *J Rheumatol*. 1999;26(11):2386-2388.
75. Romero LI, Zhang DN, Cooke JP, et al. Differential expression of nitric oxide by dermal microvascular endothelial cells from patients with scleroderma. *Vasc Med*. 2000;5(3):147-158.
76. Matucci Cerinic M, Kahaleh MB. Beauty and the beast. The nitric oxide paradox in systemic sclerosis. *Rheumatology (Oxford)*. 2002;41(8):843-847.
77. Servettaz a, Guilpain P, Goulvestre C, et al. Radical oxygen species production induced by advanced oxidation protein products predicts clinical evolution and response to treatment in systemic sclerosis. *Ann Rheum Dis*. 2007;66(9):1202-1209. doi:10.1136/ard.2006.067504.

78. Xiao Y, Li X, Cui Y, et al. Hydrogen peroxide inhibits proliferation and endothelial differentiation of bone marrow stem cells partially via reactive oxygen species generation. *Life Sci.* 2014;112(1-2):33-40. doi:10.1016/j.lfs.2014.07.016.
79. Wosniak J, Santos CXC, Kowaltowski AJ, Laurindo FRM. Cross-talk between mitochondria and NADPH oxidase: effects of mild mitochondrial dysfunction on angiotensin II-mediated increase in Nox isoform expression and activity in vascular smooth muscle cells. *Antioxid Redox Signal.* 2009;11(6):1265-1278. doi:10.1089/ars.2009.2392.
80. Nozoe M, Hirooka Y, Koga Y, et al. Mitochondria-derived reactive oxygen species mediate sympathoexcitation induced by angiotensin II in the rostral ventrolateral medulla. *J Hypertens.* 2008;26(11):2176-2184. doi:10.1097/HJH.0b013e32830dd5d3.
81. Wen H, Gwathmey JK, Xie LH. Oxidative stress-mediated effects of angiotensin II in the cardiovascular system. *World J Hypertens.* 2012;2(4):34-44. doi:10.5494/wjh.v2.i4.34.
82. Jornot L, Junod AF. Response of human endothelial cell antioxidant enzymes to hyperoxia. *Am J Respir Cell Mol Biol.* 1992;6(1):107-115. doi:10.1165/ajrcmb.6.1.107.
83. Herrick AL, Matucci Cerinic M. The emerging problem of oxidative stress and the role of antioxidants in systemic sclerosis. *Clin Exp Rheumatol.* 2001;19(1):4-8.
84. Servettaz A, Goulvestre C, Kavian N, et al. Selective oxidation of DNA topoisomerase 1 induces systemic sclerosis in the mouse. *J Immunol.* 2009;182(9):5855-5864. doi:10.4049/jimmunol.0803705.
85. Chung CP, Avalos I, Stein CM. Oxidative stress, microvascular dysfunction, and scleroderma: An association with potential therapeutic implications, a commentary on "Postocclusive reactive hyperemia inversely correlates with urinary 15-F2t-isoprostane levels in systemic sclerosis." *Free Radic Biol Med.* 2006;40(10):1698-1699. doi:10.1016/j.freeradbiomed.2006.03.001.
86. Ogawa F, Shimizu K, Muroi E, et al. Serum levels of 8-isoprostane, a marker of oxidative stress, are elevated in patients with systemic sclerosis. *Rheumatology.* 2006;45(7):815-818. doi:10.1093/rheumatology/kei012.
87. Ogawa F, Shimizu K, Hara T, et al. Autoantibody against one of the antioxidant repair enzymes, methionine sulfoxide reductase A, in systemic sclerosis: Association with pulmonary fibrosis and vascular damage. *Arch Dermatol Res.* 2010;302(1):27-35. doi:10.1007/s00403-009-0996-9.
88. Sambo P, Baroni SS, Luchetti M, et al. Oxidative stress in scleroderma: Maintenance of scleroderma fibroblast phenotype by the constitutive up-regulation of reactive oxygen species generation through the NADPH oxidase complex pathway. *Arthritis Rheum.* 2001;44(11):2653-2664. doi:10.1002/1529-0131(200111)44:11<2653::AID-ART445>3.0.CO;2-1.
89. Bruckdorfer R. The basics about nitric oxide. *Mol Aspects Med.* 2005;26(1-2):3-31. doi:10.1016/j.mam.2004.09.002.
90. Maricq HR, LeRoy EC, D'Angelo WA, et al. Diagnostic potential of in vivo capillary microscopy in scleroderma and related disorders. *Arthritis Rheum.* 1980;23(2):183-189. doi:10.1002/art.1780230208.
91. van Roon AM, Smit AJ, van Roon AM, Bootsma H, Mulder DJ. Digital ischaemia during cooling is independently related to nailfold capillaroscopic pattern in patients with Raynaud's phenomenon. *Rheumatol (United Kingdom).* 2016;55(6):1083-1090. doi:10.1093/rheumatology/kew028.
92. Kavian N, Servettaz A, Mongaret C, et al. Targeting ADAM-17/notch signaling abrogates the development of systemic sclerosis in a murine model. *Arthritis Rheum.* 2010;62(11):3477-3487. doi:10.1002/art.27626.
93. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. *World Allergy Organ J.* 2012;5(1):9-19. doi:10.1097/WOX.0b013e3182439613.
94. Bashir S, Harris G, Denman MA, Blake DR, Winyard PG. Oxidative DNA damage and cellular sensitivity to oxidative stress in human autoimmune diseases. *Ann Rheum Dis.* 1993;52(9):659-666. doi:10.1136/ard.52.9.659.

95. Boin F, Rosen A. Autoimmunity in systemic sclerosis: Current concepts. *Curr Rheumatol Rep*. 2007;9(2):165-172. doi:10.1007/s11926-007-0012-3.
96. Bordron A, Dueymes M, Levy Y, et al. The binding of some human antiendothelial cell antibodies induces endothelial cell apoptosis. *J Clin Invest*. 1998;101(10):2029-2035.
97. Hill MB, Phipps JL, Cartwright RJ, Milford Ward A, Greaves M, Hughes P. Antibodies to membranes of endothelial cells and fibroblasts in scleroderma. *Clin Exp Immunol*. 1996;106(3):491-497. doi:10.1046/j.1365-2249.1996.d01-867.x.
98. Mihai C, Tervaert JWC. Anti-endothelial cell antibodies in systemic sclerosis. *Ann Rheum Dis*. 2010;69(2):319-324. doi:10.1136/ard.2008.102400.
99. Corallo C, Franci B, Lucani B, et al. From microvasculature to fibroblasts: Contribution of anti-endothelial cell antibodies in systemic sclerosis. *Int J Immunopathol Pharmacol*. 2015;28(1):93-103. doi:10.1177/0394632015572750.
100. Praprotnik S, Blank M, Meroni PL, Rozman B, Eldor A, Shoenfeld Y. Classification of anti-endothelial cell antibodies into antibodies against microvascular and macrovascular endothelial cells: The pathogenic and diagnostic implications. *Arthritis Rheum*. 2001;44(7):1484-1494. doi:10.1002/1529-0131(200107)44:7<1484::AID-ART269>3.0.CO;2-Q.
101. Margutti P, Matarrese P, Conti F, et al. stress and endothelial cell apoptosis in immune-mediated vascular diseases and atherosclerosis Autoantibodies to the C-terminal subunit of RLIP76 induce oxidative stress and endothelial cell apoptosis in immune-mediated vascular diseases and atherosclerosis. *Hemostasis, Thromb Vasc Biol*. 2012;111(9):4559-4570. doi:10.1182/blood-2007-05-092825.
102. Danieli MG, Candela M, Ricciatti AM, et al. Antibodies to mycobacterial 65 kDa heat shock protein in systemic sclerosis (scleroderma). *J Autoimmun*. 1992;5(4):443-452. doi:0896-8411(92)90004-A [pii].
103. Ogawa F, Shimizu K, Hara T, et al. Serum levels of heat shock protein 70, a biomarker of cellular stress, are elevated in patients with systemic sclerosis: association with fibrosis and vascular damage. *Clin Exp Rheumatol*. 2008;26(4):659-662. <http://www.ncbi.nlm.nih.gov/pubmed/18799101>.
104. Brenu EW, Staines DR, Tajouri L, Huth T, Ashton KJ, Marshall-Gradisnik SM. Heat shock proteins and regulatory T cells. *Autoimmune Dis*. 2013;2013:813256. doi:10.1155/2013/813256.
105. Fujimoto M, Hamaguchi Y, Yazawa N, Komura K, Takehara K, Sato S. Autoantibodies to a collagen-specific molecular chaperone, heat-shock protein 47, in systemic sclerosis. *Clin Exp Immunol*. 2004;138(3):534-539. doi:10.1111/j.1365-2249.2004.02633.x.
106. Kiang JG, Tsokos GC. Heat shock protein 70 kDa: Molecular biology, biochemistry, and physiology. *Pharmacol Ther*. 1998;80(2). doi:10.1016/S0163-7258(98)00028-X.
107. Raska M, Weigl E. Heat shock proteins in autoimmune diseases. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2005;149(2):243-249.
108. Shukla HD, Pitha PM. Role of Hsp90 in systemic lupus erythematosus and its clinical relevance. *Autoimmune Dis*. 2012;1(1). doi:10.1155/2012/728605.
109. Kuroda K, Tsukifuji R, Shinkai H. Increased expression of heat-shock protein 47 is associated with overproduction of type I procollagen in systemic sclerosis skin fibroblasts. *J Invest Dermatol*. 1998;111(6):1023-1028. doi:10.1046/j.1523-1747.1998.00437.x.
110. Kalmar B, Greensmith L. Induction of heat shock proteins for protection against oxidative stress. *Adv Drug Deliv Rev*. 2009;61(4):310-318. doi:10.1016/j.addr.2009.02.003.
111. Pérez-Bocanegra C, Solans-Laqué R, Simeón-Aznar CP, Campillo M, Fonollosa-Pla V, Vilardell-Tarrés M. Age-related survival and clinical features in systemic sclerosis patients older or younger than 65 at diagnosis. *Rheumatology*. 2010;49(6). doi:10.1093/rheumatology/keq046.
112. Piera-Velazquez S, Jimenez SA. Role of Cellular Senescence and NOX4-Mediated Oxidative Stress in Systemic Sclerosis Pathogenesis. *Curr Rheumatol Rep*. 2014;17(1). doi:10.1007/s11926-014-0473-0.

113. Goronzy J., Weyand CM. Aging and autoimmunity. *Cell Mol Life Sci.* 2012;69(10):145-155. doi:10.1007/978-3-0346-0219-8_7.
114. Alba MA, Velasco C, Simeón CP, et al. Early- versus late-onset systemic sclerosis: differences in clinical presentation and outcome in 1037 patients. *Medicine (Baltimore).* 2014;93(2):73-81. doi:10.1097/MD.0000000000000018.
115. Shiels PG, Broen J, McGlynn L, et al. Biological ageing is a key determinant in systemic sclerosis. *J Transl Med.* 2011;9(Suppl 2):I7. doi:10.1186/1479-5876-9-S2-I7.
116. Artlett CM, Black CM, Briggs DC, Stevens CO, Welsh KI. Telomere reduction in scleroderma patients: a possible cause for chromosomal instability. *Br J Rheumatol.* 1996;35(8):732-737.
117. MacIntyre A, Brouillette SW, Lamb K, et al. Association of increased telomere lengths in limited scleroderma, with a lack of age-related telomere erosion. *Ann Rheum Dis.* 2008;67(12):1780-1782. doi:10.1136/ard.2007.086652.
118. Yamagishi M, Miyatake K, Tamai J, Nakatani S, Koyama J, Nissen SE. Intravascular ultrasound detection of atherosclerosis at the site of focal vasospasm in angiographically normal or minimally narrowed coronary segments. *J Am Coll Cardiol.* 1994;23(2):352-357. doi:10.1016/0735-1097(94)90419-7.
119. Suzuki H, Kawai S, Aizawa T, et al. Histological evaluation of coronary plaque in patients with variant angina: Relationship between vasospasm and neointimal hyperplasia in primary coronary lesions. *J Am Coll Cardiol.* 1999;33(1):198-205. doi:10.1016/S0735-1097(98)00520-8.
120. Amico D, Spadoni T, Rovinelli M, et al. Intracellular free radical production by peripheral blood T lymphocytes from patients with systemic sclerosis: role of NADPH oxidase and ERK1/2. *Arthritis Res Ther.* 2015;17(1):68. doi:10.1186/s13075-015-0591-8.
121. Hua-Huy T, Dinh-Xuan AT. Cellular and molecular mechanisms in the pathophysiology of systemic sclerosis. *Pathol Biol.* 2015;63(2):61-68. doi:10.1016/j.patbio.2015.03.003.
122. Hussain SP, Harris CC. Inflammation and cancer: An ancient link with novel potentials. *Int J Cancer.* 2007;121(11):2373-2380. doi:10.1002/ijc.23173.
123. Eckes B, Moynadeh P, Sengle G, Hunzelmann N, Krieg T. Molecular and cellular basis of scleroderma. *J Mol Med (Berl).* 2014;913-924. doi:10.1007/s00109-014-1190-x.
124. Montagnana M, Volpe A, Lippi G, et al. Relationship between matrix metalloproteinases/tissue inhibitors of matrix metalloproteinases systems and autoantibody patterns in systemic sclerosis. *Clin Biochem.* 2007;40(12):837-842. doi:10.1016/j.clinbiochem.2007.03.023.
125. Smith EA, LeRoy EC. A possible role for transforming growth factor-beta in systemic sclerosis. *J Invest Dermatol.* 1990;95(6 Suppl):1255-1275.
126. Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: The scientific foundations of cardiac repair. *J Clin Invest.* 2005;115(3):572-583. doi:10.1172/JCI200524283.
127. Shetty SS, Okada T, Webb RL, DelGrande D, Lappe RW. Functionally distinct endothelin B receptors in vascular endothelium and smooth muscle. *Biochem Biophys Res Commun.* 1993;191(2):459-464. doi:10.1006/bbrc.1993.1240.
128. Asano Y, Stawski L, Hant F, et al. Endothelial Fli1 deficiency impairs vascular homeostasis: A role in scleroderma vasculopathy. *Am J Pathol.* 2010;176(4):1983-1998. doi:10.2353/ajpath.2010.090593.
129. Jimenez S a. Role of endothelial to mesenchymal transition in the pathogenesis of the vascular alterations in systemic sclerosis. *ISRN Rheumatol.* 2013;2013:835948. doi:10.1155/2013/835948.
130. Yoshimura S, Nishimura Y, Nishiuma T, Yamashita T, Kobayashi K, Yokoyama M. Overexpression of nitric oxide synthase by the endothelium attenuates bleomycin-induced lung fibrosis and impairs MMP-9/TIMP-1 balance. *Respirology.* 2006;11(5):546-556. doi:10.1111/j.1440-1843.2006.00894.x.
131. Bogdan C. Nitric oxide and the regulation of gene expression. *Trends Cell Biol.* 2001;11(2):66-75. doi:10.1016/S0962-8924(00)01900-0.

132. Dooley A, Bruckdorfer KR, Abraham DJ. Modulation of fibrosis in systemic sclerosis by nitric oxide and antioxidants. *Cardiol Res Pract.* 2012;1(1). doi:10.1155/2012/521958.
133. Maxwell PH, Ratcliffe PJ. Oxygen sensors and angiogenesis. *Semin Cell Dev Biol.* 2002;13(1):29-37. doi:10.1006/scdb.2001.0287.
134. Beyer C, Schett G, Gay S, Distler O, Distler JH. Hypoxia. Hypoxia in the pathogenesis of systemic sclerosis. *Arthritis Res Ther.* 2009;11(2):220. doi:10.1186/ar2598.
135. Chora I, Romano E, Manetti M, et al. Evidence for a derangement of the microvascular system in patients with a very early diagnosis of systemic sclerosis. *J Rheumatol.* 2017;44(8). doi:10.3899/jrheum.160791.
136. Ruef J, Hu ZY, Yin LY, et al. Induction of vascular endothelial growth factor in balloon-injured baboon arteries. A novel role for reactive oxygen species in atherosclerosis. *Circ Res.* 1997;81(1):24-33. doi:10.1161/01.RES.81.1.24.
137. Wang Y, Zang QS, Liu Z, et al. Regulation of VEGF-induced endothelial cell migration by mitochondrial reactive oxygen species. *Am J Physiol Cell Physiol.* 2011;301(3):C695-704. doi:10.1152/ajpcell.00322.2010.
138. Kim Y-W, Byzova T V. Oxidative stress in angiogenesis and vascular disease. *Blood.* 2014;123(5):625-631. doi:10.1182/blood-2013-09-512749.
139. Ushio-Fukai M, Alexander RW. Reactive oxygen species as mediators of angiogenesis signaling. Role of NAD(P)H oxidase. *Mol Cell Biochem.* 2004;264(1-2):85-97. doi:10.1023/B:MCBI.0000044378.09409.b5.
140. Banne AF, Amiri A, Pero RW. Reduced level of serum thiols in patients with a diagnosis of active disease. *J Anti Aging Med.* 2003;6(4):327-334. doi:10.1089/109454503323028920.
141. Lau CS, O'Dowd A, Belch JJF. White blood cell activation in Raynaud's phenomenon of systemic sclerosis and vibration induced white finger syndrome. *Ann Rheum Dis.* 1992;51(2):249-252. doi:10.1136/ard.51.2.249.
142. Roon AM van, Abdulle AE, Roon AM van, et al. A pilot study on ischemia and reperfusion injury during a raynaud's attack: sequential assessment of redox stress parameters in a unique cooling and rewarming experiment. *Abstr Present EULAR Congr 14 June, Madrid, Spain.*
143. Lau CS, Bridges AB, Muir A, Scott N, Bancroft A, Belch JJ. Further evidence of increased polymorphonuclear cell activity in patients with Raynaud's phenomenon. 1992;31(6):375-380. doi:10.1093/rheumatology/31.6.375.
144. Cracowski JL, Marpeau C, Carpentier PH, et al. Enhanced in vivo lipid peroxidation in scleroderma spectrum disorders. *Arthritis Rheum.* 2001;44(5):1143-1148. doi:10.1002/1529-0131(200105)44:5<1143::AID-ANR196>3.0.CO;2-#.
145. Tufvesson E, Bozovic G, Hesselstrand R, Bjermer L, Scheja A, Wuttge DM. Increased cysteinyl-leukotrienes and 8-isoprostane in exhaled breath condensate from systemic sclerosis patients. *Rheumatology.* 2010;(49):2322-2326. doi:10.1093/rheumatology/keq271.
146. Montuschi P, Ciabattini G, Paredi P, et al. 8-Isoprostane as a biomarker of oxidative stress in interstitial lung diseases. *Am J Respir Crit Care Med.* 1998;158(5 PART I):1524-1527. doi:10.1164/ajrccm.158.5.9803102.
147. Simonini G, Pignone A, Generini S, Falcini F, Cerinic MM. Emerging potentials for an antioxidant therapy as a new approach to the treatment of systemic sclerosis. *Toxicology.* 2000;155(1-3):1-15. doi:10.1016/S0300-483X(00)00272-9.
148. Niwa Y, Somiya K, Michelson AM, Puget K. Effect of liposomal-encapsulated superoxide dismutase on active oxygen-related human disorders. A preliminary study. *Free Radic Res.* 1985;1(2):137-153. doi:10.3109/10715768509056547.
149. Correa MJU, Mariz HA, Andrade LEC, Kayser C. Oral N-acetylcysteine in the treatment of Raynaud's phenomenon secondary to systemic sclerosis: A randomized, double-blind, placebo-controlled clinical trial. *Rev Bras Reumatol.* 2014;54(6):452-458. doi:10.1016/j.rbre.2014.09.001.

150. Sambo P, Amico D, Giacomelli R, et al. Intravenous N-acetylcysteine for treatment of Raynaud's phenomenon secondary to systemic sclerosis: A pilot study. *J Rheumatol.* 2001;28(10):2257-2262.
151. Ammendola R, Ruocchio MR, Chirico G, et al. Inhibition of NADH/NADPH oxidase affects signal transduction by growth factor receptors in normal fibroblasts. *Arch Biochem Biophys.* 2002;397(2):253-257. doi:10.1006/abbi.2001.2641.
152. Rosato E, Borghese F, Pisarri S, Salsano F. The treatment with N-acetylcysteine of Raynaud's phenomenon and ischemic ulcers therapy in sclerodermic patients: A prospective observational study of 50 patients. *Clin Rheumatol.* 2009;28(12):1379-1384. doi:10.1007/s10067-009-1251-7.
153. Denton CP, Bunce TD, Dorado MB, et al. Probulcol improves symptoms and reduces lipoprotein oxidation susceptibility in patients with Raynaud's phenomenon. *Rheumatology.* 1999;38(4):309-315. doi:10.1093/rheumatology/38.4.309.
154. Cao H, Zhou X, Zhang J, et al. Hydrogen sulfide protects against bleomycin-induced pulmonary fibrosis in rats by inhibiting NF- κ B expression and regulating Th1/Th2 balance. *Toxicol Lett.* 2014;224(3):387-394. doi:10.1016/j.toxlet.2013.11.008.
155. Wang Z, Yin X, Gao L, et al. The protective effect of hydrogen sulfide on systemic sclerosis associated skin and lung fibrosis in mice model. *Springerplus.* 2016;5(1). doi:10.1186/s40064-016-2774-4.
156. Humbert M, Coghlan JG, Ghofrani H-A, et al. Riociguat for the treatment of pulmonary arterial hypertension associated with connective tissue disease: Results from PATENT-1 and PATENT-2. *Ann Rheum Dis.* 2017;76(2). doi:10.1136/annrheumdis-2015-209087.
157. Erre GL, De Muro P, Dellacà P, et al. Iloprost therapy acutely decreases oxidative stress in patients affected by systemic sclerosis. *Clin Exp Rheumatol.* 2008;26(6):1095-1098.
158. Rafikova O, Rafikov R, Kumar S, et al. Bosentan inhibits oxidative and nitrosative stress and rescues occlusive pulmonaryhypertension. *Free Radic Biol Med.* 2013;56:28-43. doi:10.1016/j.freeradbiomed.2012.09.013.

CHAPTER

Serum free thiols predict cardiovascular events and all-cause mortality in the general population: a prospective cohort study

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ABSTRACT

Objectives: Serum free thiols (R-SH, sulfhydryl groups) reliably reflect systemic oxidative stress. Since serum free thiols are rapidly oxidized by reactive species, systemic oxidative stress is generally associated with reduced serum free thiol levels. Free thiols associate with a favorable disease outcome in many patient cohorts, and the current hypothesis is that oxidative stress might also play an important role in cardiovascular disease. In this study, we aimed to establish the role of serum free thiols in the general population by investigating their relationship with the risk of cardiovascular (CV) events and all-cause mortality.

Methods: Participants (n=5,955) of the Prevention of RENal and Vascular ENd-stage Disease (PREVEND) cohort study from the general population were included. At baseline, serum levels of free thiols were quantified and adjusted to total protein levels. Protein-adjusted serum free thiol levels were studied for their associations with clinical and biochemical parameters, as well as with the risk of CV-events and all-cause mortality.

Results: The mean protein-adjusted serum free thiol level was 5.05 ± 1.02 $\mu\text{mol/g}$ of protein. Protein-adjusted serum free thiols significantly predicted the risk of CV-events, even after adjustment for potential confounding factors (hazard ratio [HR] per doubling 0.68 [95% confidence interval [CI]: 0.47-1.00], $P = 0.048$). Similarly, protein-adjusted serum free thiols were significantly predictive of the risk of all-cause mortality (HR per doubling 0.66 [95% CI 0.44-1.00], $P = 0.050$). Stratified analyses revealed lower HRs for subjects with a lower BMI, without hypertension, and without diabetes. Conversely, HRs were lower in subjects with albuminuria.

Conclusions: In this large population-based cohort study, serum free thiols significantly predicted the risk of CV-events and all-cause mortality. Our results highlight the potential significance and clinical applicability of serum free thiols since they are amendable to therapeutic intervention.

INTRODUCTION

Cardiovascular disease (CVD) is among the leading causes of morbidity and mortality globally, and during the past decade the number of CVD-associated deaths has increased by 12.5%.^{1,2} Although a large proportion of the overall cardiovascular risk can be explained by known risk factors (e.g., smoking, hypertension, obesity, diabetes, and hypercholesterolemia), the pathogenesis of CVD still remains incompletely understood.³ Current approaches to reduce the CVD burden have focused on encouraging healthier lifestyles. Other approaches have focused on the early identification of patients who are at increased risk of CVD. These latter strategies have faced multiple problems, including limited availability of non-invasive predictive biomarkers to assess the risk of cardiovascular (CV) events or mortality.

Oxidative stress is defined as the imbalance between the production of reactive oxygen species (ROS) and antioxidants. Although ROS, a collective term for oxygen-containing reactive species, are important signaling molecules regulating a variety of physiological processes, overproduction of ROS leads to cellular damage and tissue destruction. Chronic oxidative stress can easily and reliably be monitored as the depletion of serum free thiols, since they are readily oxidized by reactive species.⁴⁻⁶ In support, overproduction of ROS, as reflected by reduced levels of free thiols, is frequently observed in human disease (like in chronic kidney disease, chronic heart failure, diabetes, cancer, systemic sclerosis, and Crohn's disease) that is typically associated with oxidative stress.⁷⁻¹⁰ Free thiol groups (R-SH, sulfhydryl groups) are thought to play a protective role against oxidative stress through ROS scavenging, and are an important component of the in vivo antioxidant buffer capacity. Studies from our centre have demonstrated that higher levels of serum free thiols (i.e., lower levels of oxidative stress) are associated with favorable outcome in patients with chronic heart failure,⁷ as well as with a beneficial outcome and cardiovascular risk profile in renal transplant recipients.¹¹

Oxidative stress has been acknowledged to play an important role in CVD, and conditions promoting oxidative stress have also been associated with risk of CVD.¹² Therefore, we hypothesized that serum free thiol groups, as overall markers of systemic oxidative stress, might have merit as a read-out of cardiovascular health in the general population. Moreover, free thiols might be a valuable indicator for the risk of all-cause mortality and risk of CV-events, which could be applicable for risk stratification aimed to reduce CVD burden. Therefore, the aim of this study was to determine the predictive value of serum levels of free thiols regarding the risk of cardiovascular events and all-cause mortality in the general population.

METHODS

STUDY POPULATION

This study used data from the Prevention of RENal and Vascular ENd-stage Disease (PREVEND) cohort study.¹³ This is a large, prospective population-based cohort study with participants from the northern part of the Netherlands. The PREVEND study was set up to investigate cardiovascular and renal disease outcome focused on albuminuria as the main risk factor. From 1997 to 1998, 85,421 inhabitants aged 28-75 years from the northern parts of the Netherlands, received a questionnaire asking information about demographics, medication use, cardiovascular disease and pregnancy, including a request to supply an early morning urine sample. Participants who had a previous diagnosis of type 1 diabetes mellitus, insulin-treated type 2 diabetes mellitus and pregnant women were excluded from the study. In total, 40,856 subjects responded to the questionnaire and were analyzed for urinary albumin concentrations. Study participants having urinary albumin concentrations ≥ 10 mg/L ($n=6,000$) were invited to visit the outpatient research clinic of the University Medical Center Groningen (UMCG), as well as a random selection of participants with urinary albumin concentrations < 10 mg/L ($n=2,592$). Therefore, the PREVEND study consisted of an initial total of 8,592 participants who completed the full study program.¹⁴ However, serum samples from 6,136 participants were collected during the second visit (2001-2003, referred to as the baseline visit in the present study). Participants who had a CV event between visit 1 and visit 2 ($n=181$) were excluded from the analyses given the fact that re-events were not registered for these subjects. However, participants with a known cardiovascular disease prior to visit 2, who did not experience a CV-event between visit 1 and visit 2, were included. Therefore, a total of 5,955 participants were included in the current study. This study was approved by the Institutional Review Board (IRB) (full name in Dutch: 'Medisch Ethische Toetsingscommissie', METc) of the UMCG. The study was conducted in accordance with the principles of the Declaration of Helsinki (2013), and all study participants provided written informed consent.

DATA COLLECTION

All study participants visited the outpatient research clinic of the University Medical Center Groningen (UMCG), Groningen, the Netherlands. During the first visit, participants were requested to complete a questionnaire containing information about demographics, health status, history of cardiovascular disease (CVD), medication usage and lifestyle habits (e.g., smoking habits), and anthropometric measurements were performed as well. At the second visit, systolic and diastolic blood pressure was automatically measured every minute for a period of 8 minutes in supine position (Dinamap XL Model 9300 series device, Johnson & Johnson Medical, Tampa, FL). Blood pressure was defined as the average of the last two measurements. Furthermore, serum samples were collected and patients were asked to collect 24-h urine after they were provided with both oral and written instructions. Serum samples were stored at -80°C , while urine samples were stored at -20°C until further analysis.

LABORATORY MEASUREMENTS

High-sensitive C-reactive protein (hsCRP) and urinary albumin excretion (UAE) were measured by nephelometry (Dade Behring Diagnostics, Marburg, Germany). UAE was measured twice in two different 24-h urine collections and the average was taken for analyses. Serum total cholesterol and fasting glucose were measured by dry chemistry (Eastman Kodak, Rochester, NY, USA). Serum total protein was measured using spectrophotometry (Roche Modular, Roche, Mannheim, Germany). High-density lipoprotein (HDL) cholesterol was measured using a homogeneous method (direct HDL, Aeroset™ System, Abbott Laboratories, Abbott Park, IL, USA). Low-density lipoprotein (LDL) cholesterol was determined by the Friedewald formula (if triglycerides ≤ 4.5 mmol/L). Triglycerides were measured with an enzymatic method. Serum creatinine was measured enzymatically (Roche Modular, Roche Diagnostics, Mannheim, Germany). Serum cystatin C was measured using the Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a modular analyzer (Roche Diagnostics). Cystatin C was directly calibrated using standards from the manufacturer's supplies (according to the International Federation of Clinical Chemistry Working Group for Standardization of Serum Cystatin C).¹⁵

SERUM FREE THIOL MEASUREMENTS

Measurement of serum free thiols (R-SH, sulfhydryl groups) was performed as previously described, with minor modifications.^{16,17} After thawing, samples were 4-fold diluted using 0.1 M Tris buffer (pH 8.2). Background absorption was measured at 412 nm using the Varioskan microplate reader (ThermoScientific, Breda, the Netherlands), together with a reference measurement at 630 nm. Subsequently, 20 μ L 1.9 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB, Ellman's Reagent, CAS no. 69-78-3, Sigma Aldrich Corporation, St. Louis, MO, USA) was added to the samples in 0.1 M phosphate buffer (pH 7.0). After an incubation time of 20 min at room temperature, absorbance was measured again. Final serum free thiol concentrations were established by parallel measurement of an L-cysteine (CAS no. 52-90-4, Fluka Biochemika, Buchs, Switzerland) calibration curve (range from 15.6 μ M to 1,000 μ M) in 0.1 M Tris/10 mM EDTA (pH 8.2). Intra- and interassay coefficients of variation (CV) of serum free thiol measurements were 1.9% and 5.0%, respectively. Finally, serum free thiol concentrations were adjusted to total serum protein (measured according to standard procedures) by calculating the free thiol/total protein ratio (μ mol/g of protein). This adjustment was performed since serum proteins harbor the largest amount of free thiols and therefore greatly determine the quantity of potentially detectable free thiol groups.¹⁸

STUDY OUTCOMES AND DEFINITIONS

Estimated glomerular filtration rates (eGFR) were calculated using the combined creatinine cystatin C-based Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.¹⁹ Type 2 diabetes mellitus (T2DM) was defined as a fasting glucose level ≥ 7.0 mM or the use of antidiabetic medications according to the guidelines of the American Diabetic Association (ADA). Hypercholesterolemia was defined as a serum

total cholesterol level > 6.5 mmol/L, serum HDL-cholesterol level < 0.9 mmol/L or the use of lipid-lowering drugs at baseline. Fatal and non-fatal cardiovascular events (CV) (i.e., combined outcomes of acute myocardial infarction (AMI), acute or subacute ischemic heart disease (IHD), coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA), intracerebral hemorrhage, other intracranial hemorrhage, subarachnoid hemorrhage, occlusion and stenosis of precerebral or cerebral arteries, and other vascular interventions such as carotid desobstruction, aorta peripheral bypass surgery or percutaneous transluminal femoral angioplasty) and all-cause mortality were considered primary endpoints of the current study. These outcomes were determined by using information from the Dutch national registry of all hospital discharge diagnoses (Prismant). This information was classified in accordance with the International Statistical Classification of Diseases (ICD-10) and the International Classification of Health Interventions.²⁰ In addition, the classical 3-point major adverse cardiovascular events (3P-MACE) was considered as composite endpoint for CV events, consisting of fatal CV events, nonfatal myocardial infarction, and nonfatal stroke.²¹

STATISTICAL ANALYSIS

Data analysis was performed using the SPSS Statistics 25.0 software package (SPSS Inc., Chicago, IL, USA), Stata 15.1 (Stata Corp LLC, College Station, TX, USA), and R version 3.5.2. (Vienna, Austria). Data visualization was performed using GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA) and RStudio (version 1.2.1335; RStudio, Boston, MA). Demographic and clinical characteristics were presented as mean $\pm$ standard deviation (SD), median (interquartile range [IQR]) in case of skewed variables or proportions *n* with corresponding percentages (%). Normality was assessed and visualized using histograms and normal probability plots (Q-Q plots). Between-group comparisons for continuous variables were performed using one-way analysis of variance (ANOVA) or Kruskal-Wallis tests, while for nominal variables chi-square tests were performed, as appropriate. Protein-adjusted serum free thiol concentrations were ²log-transformed to facilitate results interpretation (per doubling). Survival distributions were assessed for tertiles of protein-adjusted serum free thiol concentrations using Kaplan-Meier curves and compared with log-rank tests. Survival time was defined from baseline (time of serum sample collection) until the date of the last examination that participants attended: the incidence of the cardiovascular (CV) event, death, or January 1, 2010 (end of follow-up). Cox proportional hazards regression analyses were performed to assess the association between protein-adjusted serum free thiols and the risk of cardiovascular events or all-cause mortality. Results were expressed as hazard ratios (HRs) with corresponding 95% confidence intervals. Univariable associations were followed by multivariable Cox regression models to adjust for potential confounding factors and stratified analyses to assess HRs across several subgroups. To evaluate discriminative capacity of Cox regression models, receiver operating characteristics (ROC) analyses were performed by calculating the Harrell C index for the various models. The Harrell C index represents the area under the ROC curve (AUC) and is compatible with time-to-event data. Finally, Cox regression analyses were repeated with restricted cubic splines with three knots

to evaluate potential non-linearity of associations between protein-adjusted serum free thiols and CV events or all-cause mortality. Non-linearity was evaluated by likelihood ratio tests, where nested models were compared using linear or linear and cubic spline terms. In addition, a sensitivity analysis was performed in which oversampling of subjects with higher urinary albumin concentrations was addressed by using design-based Cox proportional hazards regression models. Two-tailed P-values ≤ 0.05 were considered statistically significant.

SELECTION OF CONFOUNDING VARIABLES

In line with the guidelines for adjusting for confounders as described previously, we used causal models (Directed Acyclic Graphs (DAGs) and their associated theory) to distinguish the appropriate set of confounders for estimating our effect of interest.²²⁻²⁴ Based on previously established theory in our field and respecting constraints imposed by time and logic, the DAG represents the causal mechanisms we hypothesize to be underlying the variables at hand (scenario 1) (Additional file 1: Figure S1).^{9,25-31}

Arrows depict hypothesized causal (direct) effects between variables, whereas absence of an arrow between two variables represents the assumption of no such direct effect. Focusing on the effect of oxidative stress on outcome (cardiovascular events and all-cause mortality), we can now identify those variables for which conditioning in the analysis is necessary in order to obtain an un-confounded effect estimate in our statistical analysis. This is performed by determining unblocked causal paths in the graph from oxidative stress to outcome, which - when left unblocked - would bias the desired effect estimate. Consequently, we concluded that conditioning on the variables gender, age, smoking, systolic blood pressure, total cholesterol, diabetes, hsCRP (as a proxy for inflammation) in the analysis would provide an un-confounded effect estimate of oxidative stress on outcome.

As there could also be arguments that would lead the arrow pointing from hsCRP (as a proxy for inflammation) to oxidative stress to point the other way around,³² we additionally included BMI in our set of confounders to eliminate additional potential bias introduced by that scenario (scenario 2). It has to be noted that including BMI in the DAG (scenario 1) should causally have no influence on the effect estimation (Additional file 1: Figure S1).

Additional file 1: Figure S1. Directed Acyclic Graph (DAG) showing the causal paths which were hypothesized to be involved in the relationship between systemic oxidative stress and the risk of cardiovascular events and all-cause mortality. Arrows depict hypothesized causal (direct) effects between variables, whereas absence of an arrow between two variables represents the assumption of no such direct effect.

RESULTS

PATIENT CHARACTERISTICS

Baseline characteristics of the study population are presented in Table 1. The mean age was 51.6 (43.3-61.7) years, and 51% of the participants were female. Participants in the lowest tertile of protein-adjusted serum free thiols were significantly older, and had a higher body-mass index (BMI) as compared to the other groups (both $P < 0.001$). A history of cardiovascular disease was observed in 3.6% of the total cohort, and diabetes was found in 2.4% of the total number of participants. Mean protein-adjusted serum free thiol level was $5.05 \pm 1.02 \mu\text{mol/g}$ of protein (median $5.07 \mu\text{mol/g}$ of protein, full range: $1.47\text{-}9.33 \mu\text{mol/g}$). Cardiovascular diseases and diabetes were more frequently observed in subjects within the lowest free thiol tertile (both $P < 0.001$). Higher rates of mortality ($P < 0.001$) and cardiovascular events ($P < 0.001$) were more often observed in subjects within the lowest tertile of serum free thiols. Figure 1 shows the distribution of serum free thiol tertiles among survivors and non-survivors, and among subjects with and without a CV-event.

Table 1: Demographic and clinical characteristics of the study population.

	Total N=5955	T1 N=1985	T2 N=1985	T3 N=1985	P-value
Serum free thiol (μmol/g levels (adjusted for protein))	5.05 (± 1.02) μmol/g	< 4.65 μmol/g	4.65-5.46 μmol/g	> 5.46 μmol/g	
Age (years)	51.6 (43.3-61.7)	57.6 (47.8-66.6)	51.1 (43.4-59.8)	47.7 (41.1-55.3)	<0.001
Female, n (%)	3038 (51.0%)	1084 (54.6%)	1013 (51.0%)	941 (47.4%)	<0.001
Blood pressure					
SBP (mmHg)	126 (± 19)	129 (± 20)	125 (± 18)	123 (± 17)	<0.001
DBP (mmHg)	73 (± 9)	74 (± 9)	73 (± 9)	73 (± 9)	<0.001
Heart rate (bpm)	68 (± 10)	69 (± 11)	69 (± 10)	68 (± 10)	0.44
Obesity variables					
BMI (kg/m ²)	26.0 (23.6-28.9)	26.8 (24.3-29.9)	26.0 (23.6-28.7)	25.3 (23.0-28.0)	<0.001
Waist circumference (cm)	91.0 (82.0-100.0)	94.0 (85.0-102.0)	91.0 (82.0-100.0)	89.0 (81.0-98.0)	<0.001
Smoking, n (%)					<0.001
Never, n (%)	1757 (29.5%)	583 (29.4%)	578 (29.1%)	596 (30.0%)	
Current, n (%)	1655 (27.8%)	494 (24.9%)	538 (27.1%)	623 (31.4%)	
Former, n (%)	2472 (41.5%)	887 (44.7%)	848 (42.7%)	737 (37.1%)	
History of cardiovascular disease, n (%)	217 (3.6%)	100 (5.0%)	69 (3.5%)	48 (2.4%)	<0.001
Diabetes, n (%)	144 (2.4%)	76 (3.8%)	34 (1.7%)	34 (1.7%)	<0.001
Medication, n (%)					
Antihypertensive drugs, n (%)	1037 (17.4%)	481 (24.2%)	307 (15.5%)	249 (12.5%)	<0.001
Lipid lowering drugs, n (%)	371 (6.2%)	155 (7.8%)	126 (6.3%)	90 (4.5%)	<0.001
Anti-diabetic drugs, n (%)	78 (1.3%)	40 (2.0%)	19 (1.0%)	19 (1.0%)	0.006
Laboratory measurements					
Total cholesterol (mmol/L)	5.5 (± 1.0)	5.5 (± 1.0)	5.5 (± 1.0)	5.4 (± 1.1)	<0.001
HDL (mmol/L)	1.2 (1.0-1.5)	1.2 (1.1-1.5)	1.2 (1.0-1.4)	1.2 (1.0-1.4)	0.68
LDL (mmol/L)	3.4 (2.7-4.2)	3.5 (2.8-4.2)	3.3 (2.7-4.1)	3.3 (2.6-4.1)	0.20
Triglycerides (mmol/L)	1.1 (0.8-1.6)	1.1 (0.8-1.6)	1.1 (0.8-1.6)	1.0 (0.8-1.6)	0.001
hsCRP (mg/L)	1.3 (0.6-2.9)	1.6 (0.8-3.9)	1.3 (0.6-2.8)	1.0 (0.5-2.3)	<0.001
Fasting glucose (mmol/L)	4.7 (4.4-5.2)	4.8 (4.5-5.3)	4.7 (4.4-5.2)	4.7 (4.4-5.2)	<0.001
eGFR (mL/min/1.73m ²)	94.5 (82.2-104.7)	86.5 (74.7-99.0)	95.0 (83.9-104.0)	99.8 (90.1-108.2)	<0.001
Albuminuria (mg/L)	8.5 (6.0-15.0)	9.3 (6.2-19.0)	8.4 (6.0-14.0)	8.1 (5.95-13.1)	<0.001
CV events, n (%)	402 (6.8%)	184 (9.3%)	127 (6.4%)	91 (4.6%)	<0.001
Mortality, n (%)	316 (5.3%)	171 (8.6%)	85 (4.3%)	60 (3.0%)	<0.001

Data are presented as mean ± SD, proportions n with corresponding percentages (%) or median (IQR). **Bold** P-values indicate statistical significance. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitive C-reactive protein; eGFR, estimated glomerular filtration rate; CV, cardiovascular.

Figure 1 (A-B). Distributions of tertiles of protein-adjusted serum free thiol concentrations among survivor and non-survivors (A), and subjects with and without a CV-event (B). Tertile 1, protein-adjusted serum free thiol concentration range: <4.65, tertile 2, protein-adjusted serum free thiol concentration range: 4.65-5.45, tertile 3, protein-adjusted serum free thiol concentration range: >5.46.

PROTEIN-ADJUSTED SERUM FREE THIOLS AND RISK OF CARDIOVASCULAR EVENTS AND ALL-CAUSE MORTALITY

Mean follow-up of study participants was 7.7 ± 2.0 years, during which 402 (6.8%) cardiovascular events occurred. The highest frequency of cardiovascular events was observed in participants within the lowest tertile of protein-adjusted serum free thiols (184 [9.3%], $P < 0.001$). Kaplan-Meier survival analysis showed a significant difference in survival distribution between tertiles of protein-adjusted serum free thiols among participants with and without cardiovascular events (Figure 2A, $P < 0.0001$, log-rank test). Cox proportional hazards regression analyses and corresponding Harrell C indices demonstrated a statistically significant prediction of the risk of cardiovascular events (Table 2A, model 1, hazard ratio [HR] per doubling of concentration 0.39 [0.29-0.51], $P < 0.001$). After adjustment for potential confounding factors (age, sex, systolic blood pressure, BMI, total cholesterol, diabetes, current smoking, and hsCRP levels), this predictive association remained statistically significant (Table 2A, model 3, HR per doubling of concentration 0.68 [0.47-1.00], $P = 0.048$, respectively). These results remained unchanged when the 3P-MACE was considered as composite endpoint. Discriminative capacity of Cox regression models significantly improved after adjustment for potential confounding factors (Figure 3A-C).

Figure 2 (A-B). Kaplan-Meier survival distributions for tertiles of protein-adjusted serum free thiol concentrations. **(A)** Kaplan-Meier curve representing cardiovascular disease free-survival with the highest rate of cardiovascular events occurring in the lowest tertile of protein-adjusted serum free thiols (log-rank test, $P < 0.0001$). **(B)** Kaplan-Meier curve representing survival with the highest mortality rate occurring in the lowest tertile of protein-adjusted serum free thiols (log-rank test, $P < 0.0001$).

Table 2. Cox proportional hazards regression models of the association between protein-adjusted serum free thiols and potential confounding factors with (A) cardiovascular events and (B) all-cause mortality.

A: Cardiovascular events

Tertiles of protein-adjusted serum free thiols				
	HR per doubling	< 4.65 $\mu\text{mol/g}$	4.65 – 5.46 $\mu\text{mol/g}$	> 5.46 $\mu\text{mol/g}$
Model 1	0.39 (0.29-0.51), P < 0.001	1.00 (Reference)	0.66 (0.53-0.83) P < 0.001	0.47 (0.37-0.61) P < 0.001
Model 2	0.71 (0.51-0.97), P = 0.031	1.00 (Reference)	0.94 (0.75-1.19) P = 0.600	0.82 (0.63-1.07) P = 0.135
Model 3	0.68 (0.47-1.00), P = 0.048	1.00 (Reference)	1.00 (0.77-1.30) P = 0.998	0.81 (0.59-1.10) P = 0.178

B: All-cause mortality

Tertiles of protein- adjusted serum free thiols				
	HR per doubling	< 4.65 $\mu\text{mol/g}$	4.65 – 5.46 $\mu\text{mol/g}$	> 5.46 $\mu\text{mol/g}$
Model 1	0.30 (0.22-0.41), P < 0.001	1.00 (Reference)	0.47 (0.37-0.61) P < 0.001	0.34 (0.25-0.45) P < 0.001
Model 2	0.71 (0.50-1.01), P = 0.056	1.00 (Reference)	0.77 (0.59-1.00) P = 0.048	0.73 (0.54-1.00) P = 0.049
Model 3	0.66 (0.44-1.00), P = 0.050	1.00 (Reference)	0.87 (0.65-1.16) P = 0.337	0.68 (0.47-0.98) P = 0.037

Model 1: crude. Model 2: model 1, age- and sex-adjusted. Model 3 (based on DAG): model 2, hsCRP, current smoking, systolic blood pressure, total cholesterol, diabetes, and BMI. **Bold** P-values indicate statistical significance. Abbreviations: HR, hazard ratio.

Figure 3 (A-F). Receiver operating characteristics (ROC) curves with associated Harrell C indices (95% confidence interval [CI]) of the different Cox proportional hazards regression models. **(A-C)** Discriminative ability of Cox regression models for the association between protein-adjusted serum free thiols and cardiovascular events. **(D-F)** Discriminative ability of Cox regression models for the association between protein-adjusted serum free thiols and all-cause mortality.

During follow-up, 316 (5.3%) subjects died. The highest mortality rate occurred in the lowest tertile of protein-adjusted serum free thiols (171 [8.6%], $P < 0.001$). Kaplan-Meier survival analysis showed a significantly differential survival distribution between tertiles of protein-adjusted serum free thiols among survivors and non-survivors (Figure 2B, $P < 0.0001$, log-rank test). Cox proportional hazards regression analyses and corresponding Harrell C indices showed a significant inverse predictive association between protein-adjusted serum free thiol concentrations and the risk of all-cause mortality (Table 2B, model 1, HR per doubling of concentration 0.30 [0.22-0.41], $P < 0.001$). This association remained significant after adjustment for potential confounders (Table 2B, model 3, 0.66 [0.44-1.00], $P = 0.050$). Discriminative capacity of Cox regression models significantly improved after adjustment for potential confounding factors (Figure 3D-F). Restricted cubic splines showed no significant deviances from linear associations with either the incidence of CV events or all-cause mortality for protein-adjusted serum free thiol concentrations (Additional file 2: Figure S2A-B, $P = 0.258$ and $P = 0.642$, respectively). In weighted analyses, in which we accounted for the oversampling of subjects with higher urinary albumin concentrations, results (free thiols per doubling) differed slightly (CV-events: 0.65 [95% CI 0.38-1.10], all-cause mortality: 0.59 [95% CI 0.34-1.02]).

Additional file 2: Figure S2 (A-B). Protein-adjusted serum free thiol concentrations (μmol/g) show no deviations from linear associations with either **(A)** the risk for CV events or **(B)** all-cause mortality. Estimated associations were derived from the adjusted Cox proportional hazards regression analysis ([model 3](#)) based on restricted cubic splines with three knots. P-values for non-linearity were $P = 0.258$ for CV-events and $P = 0.642$ for all-cause mortality. The median of protein-adjusted serum free thiol concentrations was taken as a reference standard (5.07 μmol/g of protein). Gray-shaded areas represent 95% confidence intervals.

STRATIFIED ANALYSES

Stratified analyses for the association between protein-adjusted serum free thiols and the risk of CV events showed consistently inverse associations in various subgroups, except for associations stratified by the presence of hypertension ($P_{\text{interaction}} < 0.001$) (Table 3; Figure 4). Stratification by BMI, the presence of albuminuria, hypertension, and diabetes showed significant differences between groups. Corresponding HRs were lower for subjects with a lower BMI, without hypertension, and without diabetes. Conversely, HRs were lower in subjects with albuminuria.

Table 3. Stratified analyses for the association between protein-adjusted serum free thiols and the risk of cardiovascular (CV) events across various subgroups.

Variable	CV events (n)	Total (n)	HR*	95% CI	P _{interaction}
Overall	402	5955	0.68	0.47-1.00	0.048
Gender					
Female	87	2497	0.87	0.42-1.77	0.976
Male	221	2180	0.58	0.38-0.90	
BMI					
< 25.0	68	1910	0.53	0.23-1.21	< 0.001
> 25.0	239	2762	0.71	0.47-1.08	
Albuminuria					
No	237	4285	0.71	0.46-1.08	0.008
Yes	71	394	0.52	0.24-1.12	
Hypertension					
No	219	4143	0.55	0.36-0.86	< 0.001
Yes	94	501	1.06	0.51-2.19	
CVD history					
No	271	4526	0.69	0.46-1.04	0.327
Yes	37	155	0.47	0.18-1.21	
Diabetes					
No	289	4592	0.67	0.45-0.98	0.012
Yes	15	73	0.79	0.16-3.83	
Smoking					
No	200	3402	0.78	0.49-1.27	0.484
Yes	108	1279	0.46	0.26-0.83	
Alcohol consumption					
No	99	1134	0.72	0.38-1.36	0.911
Yes	209	3547	0.63	0.40-0.98	
Hypercholesterolemia					
No	260	4372	0.61	0.41-0.90	0.062
Yes	41	242	0.83	0.28-2.42	

Stratifications by BMI, albuminuria, hypertension, diabetes and hypercholesterolemia showed significant differences between groups. Corresponding hazard ratios (HRs) are consistently lower for subjects with a more favorable cardiovascular risk profile (i.e. lower BMI and the absence of hypertension and diabetes), whereas the presence of CVD history or albuminuria show lower HRs as well. *Adjusted for potential confounding factors (sex, age, hsCRP, current smoking, systolic blood pressure, total cholesterol, diabetes, and BMI). Abbreviations: CV, cardiovascular; HR, hazard ratio; CI, confidence interval; BMI, body-mass index; CVD, cardiovascular disease.

Figure 4. Associations between protein-adjusted serum free thiol levels and the risk of cardiovascular (CV) events across various subgroups. Hazard ratios (HRs) are shown with corresponding 95% confidence intervals (CI). HRs represent consistently inverse associations across subgroups, with the exception of stratification by the presence of hypertension ($P_{\text{interaction}} < 0.001$). Stratifications by BMI, albuminuria, hypertension, diabetes, and hypercholesterolemia show significant interactions. Corresponding HRs are consistently lower for subjects with a more favorable cardiovascular risk profile (i.e. lower BMI and the absence of hypertension and diabetes), whereas the presence of CVD history or albuminuria show lower HRs as well. *HRs adjusted for potential confounding factors (sex, age, hsCRP, current smoking, systolic blood pressure, total cholesterol, diabetes, and BMI). Abbreviations: CV-e, cardiovascular events; BMI, body mass index; eGFR, estimated glomerular filtration rate; CVD, cardiovascular disease; HR, hazard ratio; CI, confidence interval.

In subjects with a known history of CVD ($n = 217$, 3.6%), Cox proportional hazards regression analyses revealed no significant age- and sex-adjusted associations between protein-adjusted serum free thiol concentrations and the risk of CV events (Table 4A, HR per doubling of concentration: 0.52 [0.23-1.19], $P = 0.120$). However, we observed significant age- and sex-adjusted associations in this subgroup between protein-adjusted serum free thiol concentrations and the risk of all-cause mortality when comparing the upper two tertiles with the lowest tertile (Table 4B, HR per doubling of concentration, tertile 2: 0.48 [0.24-0.96], $P = 0.038$; tertile 3: 0.40 [0.16-0.99], $P = 0.047$). Although there was a large difference in sample size between subgroups of subjects with and without a history of CVD ($n = 217$, 3.6% vs. $n = 5738$, 96.4%, respectively), discriminative capacity of these models (Harrell C indices) were higher in subjects without a known history of CVD compared to subjects with a history of CVD (Figure 5).

Table 4. Cox proportional hazards regression models of the age- and sex-adjusted associations between protein-adjusted serum free thiols and the risk of (A) cardiovascular events and (B) all-cause mortality in participants with and without a history of cardiovascular disease (CVD).

A: Cardiovascular events

Tertiles of protein-adjusted serum free thiols				
	HR per doubling	< 4.65 $\mu\text{mol/g}$	4.65 – 5.46 $\mu\text{mol/g}$	> 5.46 $\mu\text{mol/g}$
Total cohort	0.71 (0.51-0.97) P = 0.031	1.00 (Reference)	0.94 (0.75-1.19) P = 0.600	0.82 (0.63-1.07) P = 0.135
No history of CVD	0.77 (0.54-1.08) P = 0.127	1.00 (Reference)	0.96 (0.75-1.23) P = 0.761	0.83 (0.62-1.09) P = 0.181
History of CVD	0.52 (0.23-1.19) P = 0.120	1.00 (Reference)	0.82 (0.42-1.61) P = 0.567	0.84 (0.38-1.86) P = 0.670

B: All-cause mortality

Tertiles of protein-adjusted serum free thiols				
	HR per doubling	< 4.65 $\mu\text{mol/g}$	4.65 – 5.46 $\mu\text{mol/g}$	> 5.46 $\mu\text{mol/g}$
Total cohort	0.71 (0.50-1.01) P = 0.056	1.00 (Reference)	0.77 (0.59-1.00) P = 0.048	0.73 (0.54-1.00) P = 0.049
No history of CVD	0.80 (0.54-1.18) P = 0.253	1.00 (Reference)	0.81 (0.61-1.09) P = 0.159	0.81 (0.58-1.12) P = 0.206
History of CVD	0.47 (0.22-1.02) P = 0.055	1.00 (Reference)	0.48 (0.24-0.96) P = 0.038	0.40 (0.16-0.99) P = 0.047

All models were age- and sex-adjusted. **Bold** P-values indicate statistical significance. Abbreviations: CVD, cardiovascular disease; HR, hazard ratio.

Figure 5. Receiver operating characteristics (ROC) curves with associated Harrell C indices (95% confidence interval [CI]) of Cox proportional hazards regression models in subjects with and without a history of cardiovascular disease (CVD). **(A-B)** Discriminative ability of Cox regression models for the association between protein-adjusted serum free thiols and cardiovascular events in both subgroups. **(C-D)** Discriminative ability of Cox regression models for the association between protein-adjusted serum free thiols and all-cause mortality in both subgroups. Abbreviations: CVD, cardiovascular disease.

DISCUSSION

In this large population-based cohort, we found that protein-adjusted serum free thiol concentrations were able to significantly predict the risk of CV-events and all-cause mortality. After adjustment for confounders, this predictive capacity of protein-adjusted serum free thiol concentrations (per doubling) regarding the risk of CV-events, as well as the risk of all-cause mortality, remained statistically significant. Collectively, our results shed light on the potential significance of serum levels of free thiols for risk stratification in primary and secondary prevention settings to reduce CVD burden in the general population.

Over the last couple of decades, increasing attention has been paid to the role of oxidative stress in CVD, since it may lead to endothelial dysfunction.³³ For instance, it was previously shown that thiols are an oxidative stress marker in subjects with an acute myocardial infarction,³⁴ and that plasma thiols are significantly lower in subjects with congestive heart

failure, as compared to healthy subjects.³⁵ In the current study, we tested the hypothesis that protein-adjusted serum free thiols, due to the ROS-scavenging properties of free thiols, are markers of the systemic redox status that might be associated with the risk of CV-events and mortality in the general population. Indeed, our results show that protein-adjusted serum free thiol concentrations (per doubling) are inversely associated with the risk of CV-events after adjustment for potential confounders. Regarding mortality, after correcting for potential confounders, we observed a significant association with protein-adjusted serum free thiols. This postulation is in line with previous findings showing that serum thiols are independently associated with CV risk-scores at population level.³⁶ As compared to this cross-sectional study, however, the current study is much larger and of longitudinal origin (10 years of follow-up), and demonstrates associations of free thiols with the occurrence of CV-events and all-cause mortality.

In our stratified analyses, the association between protein-adjusted serum free thiols and the risk of CV-events showed no significant interaction between subjects with and without CVD. Moreover, we demonstrated that the association between protein-adjusted serum free thiols and the risk of CV-events was consistently inversely associated in various subgroups, except for the association stratified by the presence of hypertension. Free thiols were found to be more negatively associated with CV-events in subgroups characterized by the absence of obesity, hypertension, and diabetes. Conversely, free thiols were more negatively associated in subjects with albuminuria. Although we cannot clearly explain these findings, a possible explanation could be that albuminuria reflects ongoing endothelial damage that leads to a stronger prediction, whereas obesity and hypertension are merely risk factors and their presence does not significantly contribute to the predictive capacity. However, free thiols were more negatively associated with CV-events in subjects without diabetes. Although it could be stated that subjects with diabetes might also show generalized manifest damage, the majority of these diabetic subjects had a relatively short disease duration. Therefore, we deem it unlikely that these subjects already had diabetes-induced microvascular complications. However, the value of free thiols in these specific subgroups requires further study as subgroup sizes were relatively unbalanced.

Free thiols (R-SH, sulfhydryl groups) are more accurately reflecting the systemic in vivo redox status as compared to many other individual oxidant or antioxidant factors and their derivatives.⁵ In the extracellular environment, thiols are an integral part of a complex and dynamic redox signaling network and possess potent antioxidant activity based on their ability to rapidly scavenge circulating reactive species.¹⁸ Extracellular thiols are predominantly embedded in plasma proteins, whereas a smaller percentage is represented by low-molecular weight (LMW) thiols, such as cysteine, homocysteine and glutathione (GSH).³⁷ Blood proteins harbor the largest amount of redox-active thiol groups. In this respect, albumin is quantitatively the most important molecule within this pool (based on the existence of its single free cysteine residue, Cys³⁴), given its abundance and because it is capable of transporting LMW thiols.¹⁸ Collectively, systemic thiols circulate in both reduced and oxidized forms and, given their rapid and potent antioxidant activity, have a major impact on the net extracellular redox equilibrium. Therefore, measuring

systemic free thiol availability may constitute a simple, robust and easily reproducible method to assess the level of systemic oxidative stress in humans. Since proteins largely determine the total amount of measurable free thiol groups, adjusting these to circulating total protein or albumin levels can be regarded as an indirect approach to more precisely reflect the total serum free thiol pool.^{7,8,10,11,38}

Due to the fact that free thiols are known to be receptive to therapeutic modulation, our results shed light on the potential development of therapeutic interventions. For instance, several thiol-based antioxidants, such as N-acetylcysteine (NAC) or glutathione (GSH), have been investigated for their potential to restore extracellular thiol homeostasis in both human and animal studies.³⁹ Administration of these substances may increase the extracellular reducing capacity, though these potential effects should be carefully examined since they may disturb the diverse physiological functions of reactive species.⁴⁰

Strengths of the present study include the size and extensive clinical characterization of our study cohort and the long follow-up duration. However, several limitations also have to be taken into account. For instance, the observational nature of the study does not allow us to infer causality between serum levels of free thiols and CV-events and all-cause mortality. In addition, given the fact that the majority of patients were Caucasians, the generalizability of our results in subjects with other ethnicities remains unknown. Likewise, the current study was carried out in the northern parts of the Netherlands, therefore, the generalizability of our results could be debated. Moreover, many arrows are absent in our Directed Acyclic Graph (DAG) for which arguments for their presence could be made (for example: socio-economic status and BMI, socio-economic status and smoking, alcohol and BMI, smoking and BMI).⁴¹⁻⁴⁴ However, adding these arrows as direct effects in Additional file 1: Figure S1 (scenario 3) would not have altered the set of confounders necessary to correct for in our analysis. In that sense, our analysis is robust to most of the underlying causal assumptions made.

In conclusion, we show that protein-adjusted serum free thiol levels are significantly predictive of the risk of CV-events and all-cause mortality in the general population. Although free thiols might harbor great potential as an easily measurable biomarker in current primary and secondary CVD prevention strategies, future prospective studies will be required to further elucidate the clinical relevance of serum free thiol levels in this context.

REFERENCES

- Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: A systematic analysis for the Global Burden of Disease Study 2010. *Lancet*. 2012;380(9859):2095-2128. doi:10.1016/S0140-6736(12)61728-0
- Wang H, Naghavi M, Allen C, et al. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet*. 2016;388(10053):1459-1544. doi:10.1016/S0140-6736(16)31012-1
- D'Agostino RB, Pencina MJ, Massaro JM, Coady S. Cardiovascular disease risk assessment: Insights from Framingham. *Glob Heart*. 2013;8(1):11-23. doi:10.1016/j.gheart.2013.01.001
- Banne AF, Amiri A, Pero RW. Reduced level of serum thiols in patients with a diagnosis of active disease. *J Anti Aging Med*. 2003;6(4):327-334. doi:10.1089/109454503323028920
- Cortese-Krott MM, Koning A, Kuhnle GGC, et al. The Reactive Species Interactome: Evolutionary Emergence, Biological Significance, and Opportunities for Redox Metabolomics and Personalized Medicine. *Antioxid Redox Signal*. 2017:684-712. doi:10.1089/ars.2017.7083
- Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol*. 2003;552(2):335-344. doi:10.1111/j.1469-7793.2003.00335.x
- Koning AM, Meijers WC, Pasch A, et al. Serum free thiols in chronic heart failure. *Pharmacol Res*. 2016;111:452-458. doi:10.1016/j.phrs.2016.06.027
- Bourgonje AR, von Martels JZH, Bulthuis MLC, et al. Crohn's Disease in Clinical Remission Is Marked by Systemic Oxidative Stress. *Front Physiol*. 2019;10:2-7. doi:10.3389/fphys.2019.00499
- Liguori I, Russo G, Curcio F, et al. Oxidative stress, aging, and diseases. *Clin Interv Aging*. 2018;(13):757-772. doi:10.2147/CIA.S158513
- Abdulle AE, van Roon AM, Smit AJ, et al. Rapid free thiol rebound is a physiological response following cold-induced vasoconstriction in healthy humans, primary Raynaud and systemic sclerosis. *Physiol Rep*. 2019;(6):e14017. doi:10.14814/phy2.14017
- Frenay ARS, de Borst MH, Bachtler M, et al. Serum free sulfhydryl status is associated with patient and graft survival in renal transplant recipients. *Free Radic Biol Med*. 2016;(93):345-351. doi:10.1016/j.freeradbiomed.2016.08.024
- Go YM, Jones DP. Cysteine/cystine redox signaling in cardiovascular disease. *Free Radic Biol Med*. 2011;50(4):495-509. doi:10.1016/j.freeradbiomed.2010.11.029
- Hillege HL, Janssen WMT, Bak AAA, et al. Microalbuminuria is common, also in a nondiabetic, nonhypertensive population, and an independent indicator of cardiovascular risk factors and cardiovascular morbidity. *J Intern Med*. 2001;249(6):519-526. doi:10.1046/j.1365-2796.2001.00833.x
- Kieneker LM, Gansevoort RT, De Boer RA, et al. Urinary potassium excretion and risk of cardiovascular events. *Am J Clin Nutr*. 2016;103(5):1204-1212. doi:10.3945/ajcn.115.106773
- Grubb A, Blirup-Jensen S, Lindström V, Schmidt C, Althaus H, Zegers I. First certified reference material for Cystatin C in human serum ERM-DA471/IFCC. *Clin Chem Lab Med*. 2010;48(11):1619-1621. doi:10.1515/CCLM.2010.318
- Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys*. 1959;82(1):70-77. doi:10.1016/0003-9861(59)90090-6
- Hu M-L, Louie S, Cross CE, Motchnik P, Halliwell B. Antioxidant protection against hypochlorous acid in human plasma. *J Lab Clin Med*. 1993;121(2):257-262.
- Turell L, Radi R, Alvarez B. The thiol pool in human plasma: The central contribution of albumin to redox processes. *Free Radic Biol Med*. 2013;65:244-253. doi:10.1016/j.freeradbiomed.2013.05.050

19. Inker LA, Schmid CH, Tighiouart H, et al. Estimating Glomerular Filtration Rate from Serum Creatinine and Cystatin C. *N Engl J Med*. 2012;(367):20-29. doi:10.1056/nejmoa1114248
20. WHO. International statistical classification of diseases and related health problems - 10th revision. *World Heal Organ*. 2011.
21. Kip KE, Hollabaugh K, Marroquin OC, Williams DO. The Problem With Composite End Points in Cardiovascular Studies. The Story of Major Adverse Cardiac Events and Percutaneous Coronary Intervention. *J Am Coll Cardiol*. 2008;51(7):701-707. doi:10.1016/j.jacc.2007.10.034
22. Pearl J. *Causality, Models, Reasoning, and Inference*. CAMBRIDGE UNIVERSITY PRESS; 2000.
23. La Bastide-Van Gemert S, van den Heuvel E. Exploring Causal Hypotheses: Breaking With Long-Standing Research Traditions. *Dev Med Child Neurol*. 2013;55(11):975-976. doi:10.1111/dmcn.12269
24. Lederer DJ, Bell SC, Branson RD, et al. Control of Confounding and Reporting of Results in Causal Inference Studies. Guidance for Authors from Editors of Respiratory, Sleep, and Critical Care Journals. *Ann Am Thorac Soc*. 2019;16(1):22-28. doi:10.1513/annalsats.201808-564ps
25. Erhardt L. Cigarette smoking: An undertreated risk factor for cardiovascular disease. *Atherosclerosis*. 2009;205(1):23-32. doi:10.1016/j.atherosclerosis.2009.01.007
26. Singh-Manoux A, Shipley MJ, Bell JA, Canonico M, Elbaz A, Kivimaki M. Association between inflammatory biomarkers and all-cause, cardiovascular and cancer-related mortality. *CMAJ*. 2017;189(10):E384-E390. doi:10.1503/cmaj.160313
27. Wonisch W, Falk A, Sundl I, Winklhofer-Roob BM, Lindschinger M. Oxidative stress increases continuously with BMI and age with unfavourable profiles in males. *Aging Male*. 2012;15(3):159-165. doi:10.3109/13685538.2012.669436
28. Münzel T, Gori T, Bruno RM, Taddei S. Is oxidative stress a therapeutic target in cardiovascular disease? *Eur Heart J*. 2010;31(22):2741-2748. doi:10.1093/eurheartj/ehq396
29. Tzoulaki I, Elliott P, Kontis V, Ezzati M. Worldwide Exposures to Cardiovascular Risk Factors and Associated Health Effects: Current Knowledge and Data Gaps. *Circulation*. 2016;133(23):2314-2333. doi:10.1161/CIRCULATIONAHA.115.008718
30. Pencina MJ, D'Agostino RB, Larson MG, Massaro JM, Vasan RS. Predicting the 30-year risk of cardiovascular disease: The framingham heart study. *Circulation*. 2009;119(24):3078-3084. doi:10.1161/CIRCULATIONAHA.108.816694
31. Liao J, Muniz-Terrera G, Scholes S, Hao Y, Chen YM. Lifestyle index for mortality prediction using multiple ageing cohorts in the USA, UK and Europe. *Sci Rep*. 2018;8(1):6644. doi:10.1038/s41598-018-24778-1
32. Salzano S, Checconi P, Hanschmann EM, et al. Linkage of inflammation and oxidative stress via release of glutathionylated peroxiredoxin-2, which acts as a danger signal. *Proc Natl Acad Sci U S A*. 2014;111(33):12157-12162. doi:10.1073/pnas.1401712111
33. Cervantes Gracia K, Llanas-Cornejo D, Husi H. CVD and Oxidative Stress. *J Clin Med*. 2017;6(2):E22. doi:10.3390/jcm6020022
34. Kundi H, Ates I, Kiziltunc E, et al. A novel oxidative stress marker in acute myocardial infarction; Thiol/disulphide homeostasis. *Am J Emerg Med*. 2015;33(11):1567-1571. doi:10.1016/j.ajem.2015.06.016
35. Belch JJ, Bridges AB, Scott N, Chopra M. Oxygen free radicals and congestive heart failure. *Br Hear J*. 1991;65(5):245-248. doi:10.1136/hrt.65.5.245
36. Mangoni AA, Zinellu A, Carru C, Attia JR, McEvoy M. Serum thiols and cardiovascular risk scores: A combined assessment of transsulfuration pathway components and substrate/product ratios. *J Transl Med*. 2013;11(99). doi:10.1186/1479-5876-11-99
37. Hortin GL, Sviridov D, Anderson NL. High-abundance polypeptides of the human plasma proteome comprising the top 4 logs of polypeptide abundance. *Clin Chem*. 2008;54(10):1608-1616. doi:10.1373/clinchem.2008.108175

38. Anraku M, Chuang VTG, Maruyama T, Otagiri M. Redox properties of serum albumin. *Biochim Biophys Acta - Gen Subj*. 2013;1830(12):5465-5472. doi:10.1016/j.bbagen.2013.04.036
39. Deneke SM. Thiol-based antioxidants. *Curr Top Cell Regul*. 2000;(36):151-180. doi:10.1016/S0070-2137(01)80007-8
40. Atkuri KR, Mantovani JJ, Herzenberg LA, Herzenberg LA. N-Acetylcysteine-a safe antidote for cysteine/ glutathione deficiency. *Curr Opin Pharmacol*. 2007;7(4):355-359. doi:10.1016/j.coph.2007.04.005
41. Abreu S, Santos R, Moreira C, Santos PC, Mota J, Moreira P. Food consumption, physical activity and socio-economic status related to BMI, waist circumference and waist-to-height ratio in adolescents. *Public Health Nutr*. 2014;17(8):1834-1849. doi:10.1017/S1368980013001948
42. Meijer E, Gebhardt WA, Van Laar C, Kawous R, Beijl SCAM. Socio-economic status in relation to smoking: The role of (expected and desired) social support and quitter identity. *Soc Sci Med*. 2016;(162):41-49. doi:10.1016/j.socscimed.2016.06.022
43. Liao C, Gao W, Cao W, et al. The association of cigarette smoking and alcohol drinking with body mass index: A cross-sectional, population-based study among Chinese adult male twins. *BMC Public Health*. 2016;(16):31. doi:10.1186/s12889-016-2967-3
44. Wang P, Abdin E, Sambasivam R, Chong SA, Vaingankar JA, Subramaniam M. Smoking and Socio-demographic correlates of BMI. *BMC Public Health*. 2016;16:500. doi:10.1186/s12889-016-3182-y

Free thiols are associated with cardiovascular events and all-cause mortality

CHAPTER 6

Rapid free thiol rebound is a physiological response following cold-induced vasoconstriction in healthy humans, primary Raynaud and systemic sclerosis

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ABSTRACT

Objectives: Raynaud's phenomenon (RP) is often the first sign of systemic sclerosis (SSc). Molecular mechanisms involved are incompletely understood, but reactive oxygen, nitrogen and sulfur species are thought to play an important role in the pathogenesis of SSc. Free thiol groups play a protective role against oxidative stress and may represent an attractive therapeutic target. We aimed to investigate the effects of hypothermia-induced vasoconstriction on the responsiveness of redox-related markers.

Methods: Thirty participants (n=10/group [SSc, primary Raynaud's phenomenon (PRP), healthy controls (HC)]) were included in the current study. Fingertip photoelectric plethysmography was performed during a standardized cooling and recovery experiment. Venous blood was collected at four predetermined time points. Free thiols, NO-derived species (nitrosyl)ated species, nitrite, nitrate, sulfate and endothelin-1 were measured.

Results: Lower baseline concentrations of free thiols were observed in PRP and SSc patients (HC: 5.87 [5.41-5.99] $\mu\text{mol/gram}$; PRP: 5.17 [4.74-5.61]; SSc 5.28 [4.75-5.80], $p=0.04$). Redox-related markers remained unchanged during cooling. However, an unexpected increase in systemic free thiol concentrations was observed in all groups during the recovery phase. The response of this marker differed between groups, with a higher increase found in SSc patients (HC $D=1.30$ [1.48-1.17]; PRP $D=1.04$ [1.06-1.03]; SSc $D=1.72$ [1.13-1.49], $p=0.04$). NO-derived species, sulfate and endothelin-1 levels remained unchanged throughout the recovery phase.

Conclusion: This exploratory study sheds light on the rapid responsiveness of systemic free thiol concentrations following reperfusion, which may reflect overall redox balance. The robust response to reperfusion in SSc patients suggests that reductive systems involved in this response are functionally intact in these patients.

INTRODUCTION

Systemic sclerosis (SSc) is a complex connective tissue disease of unknown aetiology characterized by autoimmunity and severe fibrosis in the skin and internal organs.^{1,2} Vasculopathy is the hallmark of this disease and associated with endothelial dysfunction, inflammation, and extracellular matrix remodelling in the vascular wall. Raynaud's phenomenon (RP), which is an episodic vasospastic disorder of the extremities, is the earliest recognizable clinical sign that reflects the vasculopathy in SSc.³ Patients with primary Raynaud's phenomenon (PRP), in the absence of an underlying systemic disease, lack these (specific) vascular abnormalities. Increased levels of endothelin-1 (ET-1), a potent vasoconstrictor, have previously been reported in both SSc and PRP patients.^{4,5} Due to the unavailability of effective therapy, these vascular abnormalities often lead to severe morbidity and premature mortality.⁶⁻⁸

Following Murrell's hypothesis,⁹ stating that the pathogenesis of SSc is linked to the generation of reactive oxygen species (ROS), several studies have presented evidence that overproduction of ROS— a collective term for oxygen-derived species with enhanced chemical reactivity— plays an important role in the pathogenesis of SSc.¹⁰⁻¹² Although chronically elevated levels of ROS have been suspected to prolong disease, oxidative stress (i.e., an imbalance between the production of ROS and antioxidant defences¹³) may also be an initiating factor in the development of SSc vasculopathy. In addition to the well-known damaging properties of ROS, other reactive species (i.e., reactive nitrogen species [RNS] and reactive sulfur species [RSS]) are also thought to play an important role in SSc.^{7,14} More importantly, the chemical interactions between these reactive species cause homeostatic disruption due to the production of highly reactive free radicals and other secondary metabolites.^{15,16} Therefore, these reactive species should be viewed as a unified entity, also referred to as the 'reactive species interactome'.¹⁵ In addition to the increased production of free radicals, some have shown that patients with SSc also exhibit a decreased level of antioxidants, and enzyme activities related to the defence against oxidative stress.^{10,17} More particularly, endothelial cells (ECs) were previously found to be catalase deficient and, therefore, are more prone to ROS injury.¹⁸ This deficiency may eventually initiate a self-perpetuating cycle of recurrent RP attacks, increased ROS production, ischemia/reperfusion (I/R) injury, and an inability to defend against further stress.

Free thiol (i.e., sulfhydryl groups) are thought to play a protective role against oxidative stress through ROS scavenging, and thereby function as sentinels of distant danger.¹⁵ In addition, free thiols are also believed to be part of an intricate network of the redox system which regulates crucial roles in proteins, and may lead to adjustment of protein structures and enzymatic functions according to the redox state.¹⁹ Free thiols can be oxidized by ROS and other reactive species, and are active components of antioxidant buffer capacity, implicating that their extracellular level may be a direct reflection of overall redox status.^{15,20-22} Therefore, chronic systemic oxidative stress can simply be measured as the depletion of the free thiols in serum, which mainly consists of the single free SH-group of human serum albumin.²¹ Several studies have previously demonstrated clear evidence that free thiols are an indirect measure of systemic oxidative stress.

For instance, in I/R related disorders such as myocardial stunning, researchers have previously shown a loss of sulfhydryl groups following I/R. Moreover, in other conditions (e.g., chronic heart failure and renal transplantation) thiol depletion predicted mortality and disease outcome and was associated with cardiovascular disease.^{23,24}

A better understanding of the role played by oxidative stress during a Raynaud's attack could help to identify early interventions aimed at delaying the onset of overt symptoms and/or attenuating the course of the disease. The aim of this exploratory study was threefold. First, we sought to investigate whether redox-related makers (free thiols, NO-derived species and sulfate [stable end product of hydrogen sulfide]) measured at baseline differ between groups. Second, we wanted to assess the difference in effects of hypothermia induced vasoconstriction on the responsiveness, as defined as the change (delta) during cooling and recovery, of redox related markers between SSc patients, PRP patients and healthy controls (HC). Lastly, we aimed to investigate whether there is an association between the deltas of these markers. We hypothesized that a lower steady-state concentration of free thiols would be observed in patients with SSc, due to the chronic inflammation and impaired anti-oxidative defence mechanism. In addition, we anticipated to find a difference in response to recovery in comparison to the initial vasospastic event, due to the assumption that reperfusion can be potentially more harmful than ischemia itself.

MATERIAL AND METHODS

ETHICAL APPROVAL

This study was conducted in accordance with the Declaration of Helsinki. Study approval was obtained from the local Medical Ethics Committee of the University Medical Centre Groningen (METc 2015/219), the Netherlands, and all participants provided written informed consent. This study was registered in the research database of the UMCG (NL53132.042.15).

PATIENT POPULATION

This exploratory study was conducted between 2015-2016 at the University Medical Centre Groningen (the Netherlands). Three groups of participants all aged ≥ 18 years were included, group 1 comprised of healthy controls without a history of RP (N=10), group 2 included patients with PRP (N=10), and group 3 composed of patients with RP secondary to SSc (N=10). All SSc patients fulfilled the American College of Rheumatology/ European League Against Rheumatism (ACR/EULAR) 2013 criteria.²⁵ PRP patients had negative serology, normal nailfold capillaries assessed with capillaroscopy and no clinical history of digital ulcers. Diagnosis of PRP was confirmed by the treating physician. Subjects were excluded in case of inaccessibility of the photoelectric plethysmography (PPG) measurements.

PHOTOELECTRIC PLETHYSMOGRAPHY OF THE FINGERS DURING COOLING AND RECOVERY

A standardized cooling and recovery experiment was performed (Figure 1) and concomitantly PPG was recorded as described previously.²⁶⁻²⁸ All subjects were asked to refrain from alcohol intake and smoking at least 8 hours before the assessments. Before the measurements, patients were allowed to acclimatize for half an hour in a room with a stable temperature of 23-24 °C. During the experiment, the left hand of the subject (to the level of the radiocarpal joint) was submerged in a water bath of 33°C with PPG sensors attached to each finger, enabling simultaneous measurement of perfusion in all five digits. Water was cooled every 4 minutes by 3°C, for 36 minutes in total, with water at 6°C at the end of cooling. During each step, there was a stabilisation period of 4 minutes for each temperature, after which 15 seconds of perfusion was recorded. In case of unendurable pain, the cooling experiment was discontinued and that time was marked as time point 1 (T1). After the cooling steps, the hand was taken out of the water, padded dry and then rested on a dry towel to track recovery of perfusion in the open air for 10 min (time point 2). Perfusion was recorded during the last 15 seconds of every minute during recovery. Mean ischemic time (minutes) was calculated for all fingers and was defined as the mean time of perfusion loss during cooling and recovery. PPG pulses were analysed from R-peak (detected in the ECG) up to 600 ms. The mean (SD) of the amplitude of the pulses was determined, and a signal-to-noise ratio (S/N) was calculated. Perfusion was defined as $S/N > 15$.²⁶ The experiment was deemed positive for RP when

≥ 2 fingers lost perfusion and stayed abnormal during ≥ 2 consecutive steps. Data acquisition was performed using a Biopac MP-100 system with five PPG100C amplifiers and PPG200C sensors (infrared light, 860 nm), an ECG100C amplifier with ECG-cables, an SKT100C amplifier and TSD202C temperature sensor, and AcqKnowledge 3.8.2 software (Biopac Systems Inc., Goleta (CA), USA). All signals (ECG, temperature and 5 PPG's) are sampled at 100 Hz and stored in a file for off-line processing with dedicated software to determine pulsations in each finger.

Water cooled in steps of 3 °C every 4 minutes

Figure 1: Schematic overview of the study experiment. “Cooling” refers to a temperature ramp between 33°C and 6°C implemented over 36 minutes.

CLINICAL AND LABORATORY ASSESSMENTS

The following patient characteristics were obtained: sex, age, smoking behaviour, comorbidities, body weight (kg), length (cm) and use of medication. Furthermore, the presence of digital ulcers (DU) was documented and clinical evaluations including serum autoantibodies profile, oesophageal scintigraphy and pulmonary function tests were performed in all SSc patients.

Blood was drawn from the antecubital vein of the left arm at four time points: at baseline (T0), at the end of the cooling experiment (T1), and 10 minutes (T2) and 30 minutes (T3) after start of recovery. Plasma and serum samples were stored at -80°C and thawed just before use. Prior to storing, samples were centrifuged at 1300 rpm at room temperature for 10 minutes. Markers of oxidative stress (free thiols), NO-derived species and sulfate were measured. Nitros(yl)ated species (RXNO), nitrite, nitrate and free thiols (corrected for proteins) were analysed by gas-phase chemiluminescence and ion chromatography as previously described.^{23,29,30} Serum levels of ET-1 were measured according to the manufacturer's instructions using commercially available Quantikine ELISA kits (DET10 R&D Systems, Abingdon, UK) as previously described.³¹ Plasma sulfate was measured by ion chromatography, as described earlier.³²

STATISTICAL ANALYSIS

Statistics were performed with The Statistical Product and Service Solutions (SPSS; version 22, Released 2013, IBM Corp., Armonk, NY, USA). The data of this pilot study are presented as mean $\pm$ standard deviation for normally-distributed data and median (interquartile range) for data that was non-normally distributed. Given the design of the study, the experiment was analysed in two parts: from time point 0 to time point 1 (cooling) and from time point 1 to time point 3 (recovery). Differences between groups at baseline were analysed using non-parametric test (Kruskal-Wallis, Mann-Whitney U). The main objective (difference in responsiveness of the markers between groups during cooling and recovery as measured by the delta) was assessed using paired non-parametric test (Wilcoxon signed-rank test). In addition, correlations between the calculated deltas of the markers during cooling and mean ischemic time during cooling were determined by either Pearson or Spearman correlation coefficients. In addition, we also assessed the association between the calculated deltas of the markers during recovery and mean ischemic time during recovery. A p-value of ≤ 0.05 was regarded as statistically significant.

RESULTS

PATIENT CHARACTERISTICS

In total, 32 subjects were found eligible to participate in the study. Two subjects were excluded due to an inaccessible PPG measurement, thus 30 subjects were included in the analysis. Characteristics of the study participants are displayed in Table 1. The median age of all subjects was 43 years (range 18-73) and 20 (HC n=7, PRP n=8, SSc n=5) of them were females. Raynaud's attack was successfully induced in all PRP and SSc patients, but not in the healthy controls. Three PRP patients and 7 SSc patients prematurely ended the cooling part of the experiment due to unendurable pain. Time at which unendurable pain had occurred was marked as time point 1 (end of cooling).

Table 1: Patient characteristics of healthy controls, patients with primary Raynaud's phenomenon and systemic sclerosis.

	Healthy controls N=10	Primary Raynaud phenomenon N=10	Systemic sclerosis N=10
Age (years), mean $\pm$SD	29.9 $\pm$ 2.7	47.8 $\pm$ 16.2	56.2 $\pm$ 11.5
Female gender, n	7	8	5
Weight (kg), mean $\pm$SD	71.3 $\pm$ 11.7	64.4 $\pm$ 11.9	73.3 $\pm$ 11.3
Height (cm), mean $\pm$SD	177 $\pm$ 8.7	170 $\pm$ 10.0	176 $\pm$ 5.9
Smoking, n			
Current	0	1	2
Past smoker	0	3	7
Comorbidities, n			
Hypertension	0	1	2
Hypercholesterolemia	0	0	1
Diabetes	0	1	0
ACR/EULAR score $\geq$9, n	0	0	10
Disease duration (years), median (IQR)	N/A	8.0 (4.0-17.0)	4.5 (3.0-6.3)
Abnormal NCM pattern, n	0	0	10
Positive autoantibodies, n	0	0	8
Organ involvement, n			
Pulmonal	0	0	0
Oesophageal	0	0	3
Creatinine, median (IQR)	72.1 [66.5-77.6]	72.0 [63.5-72.0]	76.2 [63.5-88.6]
eGFR, median (IQR)	94.5 [90.6-114.8]	79.3 [68.3-97.4]	86.6 [74.1-98.5]
Digital ulcers, n	0	0	6
Medication, n			
Nifedipine	0	1	2
Iloprost	0	0	2
Bosentan	0	0	3

Abbreviation: ACR/EULAR: The American College of Rheumatology/European League Against Rheumatism (EULAR); NCM: Nailfold Capillary Microscopy.

DIGITAL PERFUSION DURING STANDARDIZED COOLING AND RECOVERY EXPERIMENT

The percentage of digits with normal perfusion assessed with PPG during the cooling and recovery experiment is depicted in Figure 2. A longer duration of mean ischemic time was observed in SSc patients (30 min, 24-37 min) compared to PRP (12 min, 9-14 min, $p=0.01$) and HC (1,1 min, 0-3.7 min, $p<0.001$). Also, a considerably longer recovery time was found in the SSc group compared to the PRP group (SSc: 8 min [4-10 min]; PRP: 1.1 min [1.0-2.1 min], $p<0.001$), and about 50% of the baseline perfusion recovered 10 min after cessation of cooling stress in SSc patients.

Figure 2: Digital perfusion during cooling and recovery experiment.

Legend: Percentage of digits with normal perfusion (95% CI) assessed with photoelectric plethysmography during stepwise cooling and recovery. Lines represent healthy controls ($n=10$), patients with primary Raynaud ($n=10$) and SSc patients ($n=10$). The vertical dashed line indicates the beginning of the recovery phase. All healthy controls completed the entire cooling duration of 36 minutes. Seven primary Raynaud patients completed the cooling experiment, 2 patients discontinued after 32 minutes (9°C), and 1 patient discontinued after 12 minutes (24 °C). In addition, three systemic sclerosis patients completed the cooling experiment, 1 patient discontinued after 20 minutes (18 °C), 1 patient discontinued after 24 minutes (15 °C), 2 patients discontinued after 28 minutes (12°C), and 3 patients discontinued after 32 minutes (9°C). In addition to the cooling phase, perfusion of all participants was measured during the recovery phase.

Abbreviations: HC: healthy controls; PRP: primary Raynaud's phenomenon; SSc: systemic sclerosis.

BASELINE COMPARISON OF CIRCULATING BIOMARKERS

Baseline median levels of free thiols for the groups are illustrated in Figure 3. Significantly lower concentrations of total free thiols, indicating higher oxidative stress, were observed in PRP and SSc patients, compared to healthy controls (HC: 5.87 [5.41-5.99] $\mu\text{mol}/\text{gram}$; PRP: 5.17 [4.74-5.61] $\mu\text{mol}/\text{gram}$ protein; SSc 5.28 [4.75-5.80] $\mu\text{mol}/\text{gram}$ protein, $p=0.04$). No significant differences between PRP and SSc were observed ($p=0.59$). Although nitrate, sulfate and ET-1 tended to be higher in SSc patients, no significant differences between groups were observed in baseline levels of NO-derived species (nitrate: $p=0.23$; nitrite: $p=0.88$; RXNO: $p=0.88$, Figure 4), ET-1 ($p=0.24$, Figure 5) and sulfate ($p=0.14$, Figure 6).

Figure 3: Median (IQR) concentration of free thiols during cooling (A) and recovery (B).

Legend: Measurements at four different time points during a standardized cooling and recovery experiment in healthy controls ($n=10$ at all time points), primary Raynaud ($n=10$ during cooling, $n=9$ 10 min after start recovery and $n=8$ at the end of recovery) and systemic sclerosis patients ($n=10$ during cooling and 10 min after start recovery, $n=9$ at the end of recovery). The asterisk represents a significant difference between groups ($p<0.05$). During cooling: HC $\Delta=-0.48$ [-0.57- -0.29], PRP $\Delta=0.09$ [-0.29-0.11], SSc $\Delta=-0.48$ [-0.38- -0.41]. During recovery: HC $\Delta=1.30$ [1.48-1.17]; PRP $\Delta=1.04$ [1.06-1.03]; SSc $\Delta=1.72$ [1.13-1.49].

Figure 4: Median (IQR) concentration of NO-derived species (nitrite, nitrate and RXNO) during cooling (A) and recovery (B).

Legend: Measurements at four different time points during a standardized cooling and recovery experiment in healthy controls (n=10 at all time points), primary Raynaud patients (n=10 during cooling, n= 9 10 min after start recovery and n=8 at the end of recovery) and systemic sclerosis patients (n=10 during cooling and 10 min after start recovery, n=9 at the end of recovery). During cooling: HC: nitrate: $\Delta=0.28$ [-0.53-4.04], nitrite: $\Delta=0.01$ [0.03-0.008], RXNO: $\Delta=6.32$ [3.65-4.56]; PRP: nitrate: $\Delta=-0.09$ [-0.31-6.57], nitrite: $\Delta=0.03$ [0.0004-0.04], RXNO: $\Delta=0.84$ [1.16-4.09]; SSc: nitrate: $\Delta=5.65$ [6.03- -9.17], nitrite: $\Delta=0.03$ [0.01-0.004], RXNO: $\Delta=3.88$ [3.72-3.24]. During recovery: HC: nitrate $\Delta=6.34$ [0.18- -2.99], nitrite $\Delta=-0.05$ [-0.03- -0.04], RXNO $\Delta=-2.74$ [-2.80- -4.38]; PRP: nitrate $\Delta=0.29$ [1.07- -5.89], nitrite $\Delta=-0.03$ [0.001- -0.03], RXNO $\Delta=2.30$ [4.22-5.61]; SSc nitrate $\Delta=-7.01$ [-8.83- -8.71], nitrite $\Delta=-0.007$ [-0.02-0.02], RXNO $\Delta=-0.98$ [-1.88- -0.18].

Figure 5: Median (and IQR) concentration of endothelin-1 during cooling (A) and recovery (B).

Legend: Measurements at four different time points during a standardized cooling and recovery experiment in healthy controls (n=10 at all time points), primary Raynaud (n=10 during cooling, n= 9 10 min after start recovery and n=8 at the end of recovery) and systemic sclerosis patients (n=10 during cooling and 10 min after start recovery, n=9 at the end of recovery). During cooling: HC: $\Delta=-0.18$ [-0.25- -0.26]; PRP: $\Delta=-0.37$ [-0.28- -0.31]; SSc: $\Delta=-0.56$ [-0.34- -0.86]. During recovery: HC $\Delta=0.33$ [-0.05-0.84]; PRP $\Delta=0.79$ [0.64-1.02]; SSc $\Delta=0.64$ [0.52-3.63].

Figure 6: Median (and IQR) concentration of sulfate during cooling (A) and recovery (B).

Legend: Measurements at four different time points during a standardized cooling and recovery experiment in healthy controls ($n=10$ at all time points), primary Raynaud ($n=10$ during cooling, $n=9$ 10 min after start recovery and $n=8$ at the end of recovery) and systemic sclerosis patients ($n=10$ during cooling and 10 min after start recovery, $n=9$ at the end of recovery). During cooling: HC: $\Delta=10.80$ [5.25-9.55]; PRP: $\Delta=4.45$ [2.35-13.28]; SSC: $\Delta=1.25$ [3.93-0.95]. During recovery: HC $\Delta=-10.70$ [-8.42- -11.65]; PRP $\Delta=5.55$ [6.09- -7.29]; SSC $\Delta=-10.50$ [-22.95-11.25].

DIFFERENCES IN RESPONSIVENESS OF THE MARKERS BETWEEN GROUPS DURING COOLING (TIME POINT 0 TO TIME POINT 1) AND DURING RECOVERY (TIME POINT 1 TO TIME POINT 3)

During cooling, free thiols, NO-derived species, sulfate and ET-1 remained unchanged. As shown in figures 3, 4 and 5, the deltas of free thiols, NO-derived species and ET-1 were comparable between groups (free thiols $p=0.27$; nitrate $p=0.64$; nitrite $p=0.99$; RXNO $p=0.48$, ET-1 $p=0.77$). In contrast, circulating sulfate concentrations were significantly different between groups, and the highest delta was observed in the healthy controls ($p=0.03$, figure 6).

During the recovery phase, we found that free thiol concentrations not only recover during reperfusion, but reach levels that exceed baseline concentrations. This finding was coincided with stable concentrations of the gasotransmitter end-products nitrite and nitrate (in part, originating from NO) and sulfate (related to H_2S). Moreover, the delta of free thiol concentrations were significantly different between groups (figure 3, $p=0.04$). However, those of the NO-derived species, ET-1 and sulfate were comparable between groups (nitrate $p=0.68$; nitrite $p=0.89$; RXNO $p=0.06$; sulfate $p=0.31$; ET-1 $p=0.25$).

CORRELATIONS BETWEEN LABORATORY MARKERS AND MEAN ISCHEMIC TIME DURING COOLING AND RECOVERY

We investigated the correlation between the delta of these markers and mean ischemic time during cooling and recovery, but found no significant correlations. Furthermore, correlations between the markers were assessed during cooling and recovery. During cooling, we observed a positive correlation between delta nitrate and nitrite ($r=0.54$, $p<0.01$), delta sulfate and nitrite ($r=0.39$, $p=0.03$) and delta sulfate and free thiols ($r=0.48$, $p<0.01$). During recovery, only the positive correlation between delta nitrate and nitrite was found to be significant ($r=0.44$, $p=0.02$).

DISCUSSION

This pilot study demonstrated that circulating free thiol concentrations are lower at baseline in PRP and SSc patients compared to HC. Moreover, we observed a rapid increase of free thiols in all groups during recovery following transient hypothermia. Our findings coincided with stable concentrations of the gasotransmitter end-products nitrite and nitrate (in part, originating from NO) and sulfate (related to H_2S). These results not only suggest that RP patients (i.e., primary and secondary) have chronically increased levels of oxidative stress, but also indicate that free thiol concentrations are rapidly elevated following transient hypothermia-induced vasoconstriction. The latter was an unexpected finding that warrants further investigation.

Raynaud's phenomenon in SSc patients often precedes the onset of other symptoms (e.g., organ involvement) by several years. Although primary and secondary RP share some similarities from a pathophysiological point of view, secondary RP is more likely to be associated with severe complications of vascular insufficiency due to endothelial abnormalities.³³⁻³⁷ In support, the potent vasoconstrictor ET-1 was previously suggested to play an important role in the pathogenesis of SSc vasculopathy.^{4,38,39} Although we did observe higher concentration of ET-1 in SSc patients, this marker remained stably elevated throughout the experiment. This finding is in accordance with a previous study conducted by Smyth and colleagues (2000) who did not find clear differences during a cooling experiment.⁴⁰ Collectively, these findings suggest that ET-1 is not involved in changes in vascular tone during transient hypothermia.

The current study showed a significantly lower baseline level of free thiols in both PRP and SSc patients as compared to HC, which may be indicative of chronically increased levels of oxidative stress. Oxidative stress arises when the production of ROS outweighs the buffering capacity of antioxidant defences, eventually leading to cellular damage.¹³ Following oxidation, serum thiols are not easily reduced compared to their intracellular counterparts, indicating that they are a relatively stable reflection of the systemic redox status. Consistent with this assumption, a marked reduction of serum protein thiols was previously observed in active SSc.^{21,41,42} This difference could also be explained by the difference in smoking behaviour between the groups. For instance, a current hypothesis

is that the damaging properties of smoking can even occur after cessation of smoking. Therefore, SSc patients may experience prolonged oxidative-stress induced damage even after cessation of smoking.

Interestingly, our analyses revealed an additional unexpected biomarker feature, i.e., a significant increase in concentration of free thiols during the recovery phase in all groups. Specifically, we here demonstrate that free thiol concentrations not only recover during reperfusion, but reach levels that exceed baseline concentrations. This increase in free thiols (we here define as 'thiol rebound') could be explained by several mechanisms. First, our findings may be the result of rapid reversibility and dynamic nature of processes related to oxidative thiol modification, which lowers the availability of total free thiols (reduced thiols) in the circulation. In a previously conducted experimental study in mice, the protective mechanism in endothelial function following I/R of thioredoxin, a disulfide-reducing protein, was shown.⁴³ A similar mechanism could also play a role in hypothermia induced Raynaud's attack. Secondly, this rapid responsiveness of free thiols may be the result of a physiological acute response to reperfusion, which may alter the oxidative balance. This hypothesis is supported by the fact that this thiol rebound phenomenon was also observed in healthy controls. Third, as overall thiol redox status is presumably affected by NO-derived species,¹⁵ it is conceivable that NO plays an important role in the processes related to thiol modification, that could have led to the previously mentioned thiol rebound. However, we have to address the fact that no significant increase of nitrate, or any other NO-derivative measured was observed in healthy controls and PRP patients, despite the presence of the thiol rebound during reperfusion. This observation may indicate that free thiols can be produced/modified independently of NO-derived species.

Hydrogen sulfide (H₂S), which is one of the three main gasotransmitters, has been reported to have vasodilator and antioxidant capacity. Therefore, a current hypothesis is that H₂S may play a more prominent role in the development SSc vasculopathy. In this current study we investigated the effect of hypothermia on plasma concentrations of sulfate, which is a stable end product of H₂S. We observed a trend in elevated plasma levels of sulfate in SSc patients throughout the entire experiment. Urinary sulfur metabolites, such as sulfate, were previously found to be associated with renal function, favourable cardiovascular risk profile and an improved survival in renal transplant recipients.⁴⁴ However, as none of the participants had an impaired kidney function our findings are unlikely explained by disturbances in kidney function. In addition, as we also observed a trend towards increased levels of both nitrate and sulfate in SSc patients, it is plausible that these markers of the redox system interact with one another through the same pathway; underlining the complexity of the redox interactome. The fact that both chemical compounds are known dietary constituents complicates the interpretation of these results inasmuch as we cannot exclude that differences in the consumption of nitrate and sulfate containing nutrients may have contributed to the observed changes. Clarification of the interaction of these signalling molecules in the pathogenesis of PRP and SSc awaits further studies.

The present exploratory study has some limitations. First, it could be argued that the ageing process (i.e., older age) itself potentially influences the level of oxidative stress. For instance, several studies have shown that ageing cells accumulate increased levels of oxidant-damaged DNA, which may further increase the level of oxidative stress. Therefore, the large age-range could potentially affect the baseline level of free thiols. Despite this difference in age between the groups, we have demonstrated that all three groups show the “thiol rebound” phenomenon. This finding may suggest that the response of free thiol level following hypothermia induced vasoconstriction, is not influenced by age. However, in order to investigate the effect of age on the redox status large age-matched control-groups have to be included. Second, we acknowledge the fact that the differences in gender between groups may play an important role in the oxidative balance. Given the small number of males included in this study, we were unable to demonstrate gender differences. However, this information could give us valuable insight that could potentially clarify the complexity of the redox system, and, therefore, future studies should investigate these gender differences. Third, other factors such as blood pressure, cholesterol and co-morbidities were not assessed in the current study. However, given the fact that the majority of the participants, did not show signs of hypertension or hypercholesterolemia 1 year prior and/or 1 year after the experiment (as assessed by the caregiving rheumatologist or internist), it would be unlikely that the inclusion of these factors would influence our result. Fourth, patients were not asked to discontinue their medication. As many of the vasoactive medication may alter the oxidative balance, it is possible that the ‘thiol rebound’ phenomenon as observed in SSc patients could potentially be even higher. Fifth, due to the small sample size it was not feasible to analyse differences between SSc patients with reperfusion and those with prolonged ischemia. However, based on the results of this study it is possible to calculate effect sizes, and this will assist future studies in their sample size calculation. Sixth, some patients did not complete the cooling experiment because of unendurable pain and objective documentation of the development of ischemia was lacking in these patients. However, the majority of these patients were SSc patients, and no one in the healthy control group prematurely discontinued the experiment. It is therefore conceivable that these SSc patients prematurely discontinued the experiment due to an RP attack. Despite these limitations, we were able to demonstrate clear differences in the levels of circulating free thiols during the course of a unique cooling and recovery experiment.

In conclusion, this study demonstrates decreased circulating free thiol concentrations in primary RP and SSc patients as compared to healthy controls. Moreover, an unexpected rapid increase of free thiols was present in all individuals during the reperfusion phase of the standardized cooling experiment. The rapid responsiveness of this redox related marker demonstrates the dynamic nature of this seemingly simple biomarker and may be the result of a physiological acute response to reperfusion, which may alter the oxidative balance. The observed response to reperfusion in SSc patients suggests that systems involved in the thiol responsiveness are functionally intact in these patients. Whether a further enhancement of this response, through therapeutic intervention, confers additional protection in SSc and RP remains to be investigated.

REFERENCES

1. LeRoy EC, Medsger J. Criteria for the classification of early systemic sclerosis. *J Rheumatol*. 2001;28(7):1573-1576.
2. Denton CP, Khanna D. Systemic sclerosis. *Lancet*. 2017;390(10103):1685-1699. doi:10.1016/S0140-6736(17)30933-9
3. Herrick AL. Pathogenesis of Raynaud's phenomenon. *Revmatologija*. 2005;13(3):62-65. doi:10.1093/rheumatology/keh552
4. Mostmans Y, Cutolo M, Giddelo C, et al. The role of endothelial cells in the vasculopathy of systemic sclerosis: A systematic review. *Autoimmun Rev*. 2017;16(8):774-786. doi:10.1016/j.autrev.2017.05.024
5. Zamora MR, O'Brien RF, Rutherford RB, Weil JW. Serum endothelin-1 concentrations and cold provocation in primary Raynaud's phenomenon. 1990;(336):1144-1147.
6. Leroy EC, Black C, Fleischmajer R, et al. Scleroderma (Systemic Sclerosis): Classification, Subsets and Pathogenesis. *J Rheumatol*. 1988;15(2):202-205.
7. Matucci Cerinic M, Kahaleh MB. Beauty and the beast. The nitric oxide paradox in systemic sclerosis. *Rheumatology (Oxford)*. 2002;41(8):843-847.
8. Herrick AL, Hollis S, Schofield D, et al. A double-blind placebo-controlled trial of antioxidant therapy in limited cutaneous systemic sclerosis. *Clin Exp Rheumatol*. 2000;18(3):349-356.
9. Murrell DF. A radical proposal for the pathogenesis of scleroderma. *J Am Acad Dermatol*. 1993;28(1):78-85. doi:10.1016/0190-9622(93)70014-K
10. Herrick AL, Matucci Cerinic M. The emerging problem of oxidative stress and the role of antioxidants in systemic sclerosis. *Clin Exp Rheumatol*. 2001;19(1):4-8.
11. Ogawa F, Shimizu K, Muroi E, et al. Serum levels of 8-isoprostane, a marker of oxidative stress, are elevated in patients with systemic sclerosis. *Rheumatology*. 2006;45(7):815-818. doi:10.1093/rheumatology/keh012
12. Sambo P, Baroni SS, Luchetti M, et al. Oxidative stress in scleroderma: Maintenance of scleroderma fibroblast phenotype by the constitutive up-regulation of reactive oxygen species generation through the NADPH oxidase complex pathway. *Arthritis Rheum*. 2001;44(11):2653-2664. doi:10.1002/1529-0131(200111)44:11<2653::AID-ART445>3.0.CO;2-1
13. Jones DP, Sies H. The Redox Code. *Antioxid Redox Signal*. 2015;23(9):734-746. doi:10.1089/ars.2015.6247
14. Wang Z, Yin X, Gao L, et al. The protective effect of hydrogen sulfide on systemic sclerosis associated skin and lung fibrosis in mice model. *Springerplus*. 2016;5(1). doi:10.1186/s40064-016-2774-4
15. Cortese-Krott MM, Koning A, Kuhnle GGC, et al. The Reactive Species Interactome: Evolutionary Emergence, Biological Significance, and Opportunities for Redox Metabolomics and Personalized Medicine. *Antioxid Redox Signal*. 2017:684-712. doi:10.1089/ars.2017.7083
16. Nagpure B V., Bian J-S. Interaction of Hydrogen Sulfide with Nitric Oxide in the Cardiovascular System. *Oxid Med Cell Longev*. 2016;2016:1-16. doi:10.1155/2016/6904327
17. Ogawa F, Shimizu K, Hara T, et al. Autoantibody against one of the antioxidant repair enzymes, methionine sulfoxide reductase A, in systemic sclerosis: Association with pulmonary fibrosis and vascular damage. *Arch Dermatol Res*. 2010;302(1):27-35. doi:10.1007/s00403-009-0996-9
18. Jornot L, Junod AF. Response of human endothelial cell antioxidant enzymes to hyperoxia. *Am J Respir Cell Mol Biol*. 1992;6(1):107-115. doi:10.1165/ajrcmb/6.1.107
19. Go YM, Jones DP. Cysteine/cystine redox signaling in cardiovascular disease. *Free Radic Biol Med*. 2011;50(4):495-509. doi:10.1016/j.freeradbiomed.2010.11.029
20. Turell L, Radi R, Alvarez B. The thiol pool in human plasma: The central contribution of albumin to redox processes. *Free Radic Biol Med*. 2013;65:244-253. doi:10.1016/j.freeradbiomed.2013.05.050

21. Banne AF, Amiri A, Pero RW. Reduced level of serum thiols in patients with a diagnosis of active disease. *J Anti Aging Med.* 2003;6(4):327-334. doi:10.1089/109454503323028920
22. Chung H., Wang SB, Venkatraman V, Murray CI, Van Eyk JE. Cysteine Oxidative Post-translational Modifications: Emerging Regulation in the Cardiovascular System. *Circ Res.* 2013;112(2):382-392. doi:10.1021/nl061786n.Core-Shell
23. Koning AM, Meijers WC, Pasch A, et al. Serum free thiols in chronic heart failure. *Pharmacol Res.* 2016;111:452-458. doi:10.1016/j.phrs.2016.06.027
24. Koning AM, Frenay ARS, Leuvenink HGD, Van Goor H. Hydrogen sulfide in renal physiology, disease and transplantation - The smell of renal protection. *Nitric Oxide - Biol Chem.* 2015;46:37-49. doi:10.1016/j.niox.2015.01.005
25. Van Den Hoogen F, Khanna D, Fransen J, et al. 2013 classification criteria for systemic sclerosis: An american college of rheumatology/European league against rheumatism collaborative initiative. *Arthritis Rheum.* 2013;65(11):2737-2747. doi:10.1002/art.38098
26. van Roon AM, Smit AJ, van Roon AM, Bootsma H, Mulder DJ. Digital ischaemia during cooling is independently related to nailfold capillaroscopic pattern in patients with Raynaud's phenomenon. *Rheumatol (United Kingdom).* 2016;55(6):1083-1090. doi:10.1093/rheumatology/kew028
27. Wouda AA. Raynaud' s phenomenon. *Cardiovasc Res.* 1977;35(1):175. doi:10.1016/S0008-6363(97)00094-1
28. Suichies HE, Aarnoudse JG, Wouda AA, Jentink HW, de Mul FF, Greve J. Digital blood flow in cooled and contralateral finger in patients with Raynaud's phenomenon. Comparative measurements between photoelectrical plethysmography and laser Doppler flowmetry. *Angiology.* 1992;43(2):134-141. doi:10.1177/000331979204300207
29. Rassaf T, Bryan NS, Kelm M, Feelisch M. Concomitant presence of N-nitroso and S-nitroso proteins in human plasma. *Free Radic Biol Med.* 2002;33(11):1590-1596. doi:10.1016/S0891-5849(02)01183-8
30. Feelisch M, Rassaf T, Mnaimneh S, et al. Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. *FASEB J.* 2002;16(13):1775-1785. doi:10.1096/fj.02-0363com
31. Jongman RM, Zijlstra JG, Kok WF, et al. Off-Pump CABG Surgery Reduces Systemic Inflammation Compared With On-Pump Surgery but Does Not Change Systemic Endothelial Responses. *Shock.* 2014;42(2):121-128. doi:10.1097/SHK.0000000000000190
32. Koning AM, Meijers WC, Minović I, et al. The fate of sulfate in chronic heart failure. *Am J Physiol - Hear Circ Physiol.* 2017;312(3):H415-H421. doi:10.1152/ajpheart.00645.2016
33. Flavahan NA. A vascular mechanistic approach to understanding Raynaud phenomenon. *Nat Rev Rheumatol.* 2015;11(3):146-158. doi:10.1038/nrrheum.2014.195
34. Herrick AL. Pathogenesis of Raynaud's phenomenon. *Rheumatology.* 2005;44(5):587-596. doi:10.1093/rheumatology/keh552
35. Herrick AL. The pathogenesis, diagnosis and treatment of Raynaud phenomenon. *Nat Rev Rheumatol.* 2012;8(8):469-479. doi:10.1038/nrrheum.2012.96
36. Houtman PM, Kallenberg CG, Wouda AA, The TH. Decreased nailfold capillary density in Raynaud's phenomenon: a reflection of immunologically mediated local and systemic vascular disease? *Ann Rheum Dis.* 1985;44:603-609. doi:10.1136/ard.44.9.603
37. Herrick AL. Therapeutic implications from the pathogenesis of Raynaud's phenomenon. *Expert Rev Clin Immunol.* 2017;00(00):1-13. doi:10.1080/1744666X.2017.1279052
38. Silveri F, De Angelis R, Poggi A, et al. Relative roles of endothelial cell damage and platelet activation in primary Raynaud's phenomenon (RP) and RP secondary to systemic sclerosis. *Scand J Rheumatol.* 2001;30(5):290-296. doi:10.1080/030097401753180372

39. Avouac J, Vallucci M, Smith V, et al. Correlations between angiogenic factors and capillaroscopic patterns in systemic sclerosis. *Arthritis Res Ther*. 2013;15(2):R55. doi:10.1186/ar4217
40. Smyth AE, Bell AL, Bruce IN, McGrann S, Allen JA. Digital vascular responses and serum endothelin-1 concentrations in primary and secondary Raynaud's phenomenon. *Ann Rheum Dis*. 2000;59(11):870-874. doi:10.1136/ard.59.11.870
41. Lau CS, O'Dowd A, Belch JJF. White blood cell activation in Raynaud's phenomenon of systemic sclerosis and vibration induced white finger syndrome. *Ann Rheum Dis*. 1992;51(2):249-252. doi:10.1136/ard.51.2.249
42. Giovannetti A, Gambardella L, Pietraforte D, et al. Red Blood Cell Alterations in Systemic Sclerosis: a Pilot Study. *Cell Physiol Biochem*. 2012;(30):418-427. doi:10.1159/000339035
43. Subramani J, Kundumani-Sridharan V, Hilgers RHP, Owens C, Das KC. Thioredoxin uses a GSH-independent route to deglutathionylate endothelial nitric-oxide synthase and protect against myocardial infarction. *J Biol Chem*. 2016;291(45):23374-23389. doi:10.1074/jbc.M116.745034
44. van den Berg E, Pasch A, Westendorp WH, et al. Urinary Sulfur Metabolites Associate with a Favorable Cardiovascular Risk Profile and Survival Benefit in Renal Transplant Recipients. *J Am Soc Nephrol*. 2014;25(6):1303-1312. doi:10.1681/ASN.2013050497

Rapid free thiol rebound is a physiological response following cold-induced vasoconstriction

7

CHAPTER

Hydrogen sulfide: A therapeutic option in systemic sclerosis

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ABSTRACT

Systemic sclerosis (SSc) is a lethal disease characterized by auto-immunity, vascular injury and progressive fibrosis of multiple organ systems. Despite the fact that the exact etiology of SSc remains unknown, oxidative stress has been associated with a large range of SSc-related complications. In addition to the well-known detrimental properties of reactive oxygen species (ROS), gasotransmitters (e.g., nitric oxide (NO), carbon monoxide (CO), hydrogen sulfide (H_2S)) are also thought to play an important role in SSc. Accordingly, the diverse physiologic actions of NO and CO, and their role in SSc have been previously studied. Recently, multiple studies have also shown the importance of the third gasotransmitter H_2S in both vascular physiology and pathophysiology. Interestingly, homocysteine (which is converted into H_2S through the transsulfuration pathway) is often found to be elevated in SSc patients; suggesting defects in the transsulfuration pathway. Hydrogen sulfide, which is known to have several effects including a strong antioxidant and vasodilator effect, could potentially play a prominent role in the initiation and progression of vasculopathy. A better understanding of the actions of gasotransmitters like H_2S , in the development of SSc-related vasculopathy, could help create early interventions to attenuate the disease course. This paper will review the role of H_2S in vascular (patho-)physiology and potential disturbances in SSc. Moreover, current data from experimental animal studies will be reviewed. Lastly, we will evaluate potential interventional strategies.

INTRODUCTION

Systemic sclerosis (SSc) is a severe progressive fibrotic connective tissue disease that often affects multiple systems.^{1,2} The disease is characterized by chronic inflammation, autoimmunity, and vasculopathy which all lead to substantial disability, high morbidity and mortality rates, and a decreased quality of life.³ Reported prevalence rates for SSc range from 30 to 300 cases per 1 million persons, and the age of onset is approximately 50 years.⁴⁻⁶ A variety of disabling complications (e.g., skin fibrosis, gastrointestinal dysmotility, scleroderma renal crisis (SRC), and lung involvement) occur in the early stages of the disease. Despite many efforts, the pathogenesis still remains unknown and effective disease-modifying treatment is currently unavailable. In addition, current treatment options (such as immunosuppressants) can cause a variety of adverse effects, including bone marrow depression, impaired hepatic and renal function and infections. Therefore, new (low-risk) therapy options need to be found.

Endothelial dysfunction is a main event in the pathogenesis of SSc,⁷ and the vascular bed is a major target for immune-inflammatory injury. The vascular injury (i.e., vasculopathy) is characterized by dysfunction of endothelial cells (ECs), inflammation, and vascular wall remodeling.⁸ Raynaud's phenomenon (RP), which manifest as episodic vasospastic events leading to discoloration of the extremities, is the most recognizable and primary clinical sign that reflects the vascular injury.⁹ Raynaud's phenomenon often precedes visceral disease by several years and, therefore, RP is a crucial part of the important criteria for the diagnosis of early SSc (VEDOSS criteria).² Following RP, the vasculopathy often progresses and becomes clinically evident as necrotic lesions of the fingertips (i.e., digital ulcerations), SRC, and pulmonary arterial hypertension (PAH).¹⁰ A current hypothesis is that early detection of SSc may allow an adequate start of appropriate therapeutic regimen to arrest disease progression.

The exact etiology of SSc remains unknown, but some studies have suggested that gasotransmitters play an important role in its pathogenesis. Gasotransmitters, which are endogenously produced gaseous signaling molecules, play a major role in several physiological processes ranging from vessel tone regulation, modulation of the immune system, and protection against oxidative stress.^{11,12} To date, three main gasotransmitters have been discovered namely, hydrogen sulfide (H_2S), carbon monoxide (CO), and nitric oxide (NO).¹³ As these gases perform critical roles, a deviation of their normal level may be associated with the occurrence of several diseases ranging from neurodegenerative diseases to auto-immune diseases. Interestingly, some reports have stated that SSc patients show a decreased level of H_2S . Moreover, the beneficial effects of H_2S in scleroderma-like experimental animal study have also been proven. Therefore, assessment of the role played by H_2S in SSc-related vasculopathy could lead to a better understanding of this disease, which could subsequently help to create early interventions to attenuate the disease course. This review focuses on the role of H_2S the pathogenesis of SSc-related vasculopathy in relation to its functional properties in the healthy vascular system. Furthermore, we evaluate the effects of H_2S -based therapeutic interventions.

ROLE OF NITRIC OXIDE AND CARBON MONOXIDE IN SYSTEMIC SCLEROSIS

The role of NO in SSc has been extensively discussed elsewhere,¹⁴⁻¹⁹ and, therefore, will be shortly mentioned here. The free radical gas NO is synthesized from the amino acid L-arginine by a family of isoenzymes, termed nitric oxide synthases (NOS).^{20,21} There are three known isoforms, namely endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Endothelial NOS is the major isoform regulating vascular function, and is primarily expressed in ECs, cardiomyocytes, neurons and hepatocytes.²² Induction of iNOS occurs usually in response to infection and chronic inflammation,²³ and is especially found in immunological cells such as macrophages and neutrophils.^{24,25} Nitric oxide may stimulate soluble guanylyl cyclase (sGC) in vascular smooth muscle cells (VSMCs) to induce the formation of cyclic guanosine monophosphate (cGMP).^{26,27} Subsequently, the formation of cGMP activates protein kinase G (PKG),²⁷ which leads to the reduction of cytosolic Ca^{2+} and reduction of the sensitivity of smooth muscle cells (SMCs) to Ca^{2+} . As a result of this process, the intracellular concentration of calcium decreases and vasorelaxation is promoted.^{12,28,29} Endothelial dysfunction is believed to be a main target in SSc. Although several mechanisms have been proposed which play an important role in this process, the exact role of NO still remains incompletely understood as both protective and harmful effect of NO have previously been described.¹⁴ It was previously suggested that a decreased release of NO, which may lead to impaired endothelial-dependent vasodilatation, may play an important role in the pathogenesis of the SSc-related vasculopathy. In support, several studies reported on a decreased level of NO release.^{30,31} Subsequently, this may promote the development of vasculopathy by inducing recurrent RP attacks, increasing vascular wall thickness, and by enhancing the platelet aggregation. Moreover, NO is also believed to protect the endothelium from oxidative stress by acting as a free radical scavenger, and inhibiting lipid peroxidation.^{32,33} In contrast, NO is also believed to have several harmful effects. For instance, it was previously reported that iNOS expression, which is often considered as a harmful enzyme,³⁴ is up-regulated in affected skin, fibroblast, and endothelial cells.^{15,35} Interestingly, it has been proposed that with the progression of SSc the NO production, which is primarily produced by eNOS in the early stages of the disease, is replaced by iNOS in endothelial cells and fibroblast.^{15,35} This finding may indicate that the increased levels of NO which were previously reported in SSc,³⁶ may be induced by increased expression of mainly iNOS. These conflicting results indicate that the exact role of NO in SSc still remains complex, and further studies should be conducted in order to clarify the role of NO in the pathogenesis of SSc.

Although the role of CO in SSc is less known, previous studies have suggested that CO may play an important role in several vascular diseases with a similar pathology as SSc. Carbon monoxide is generated by haem oxygenases (HO), namely HO-1 and HO-2 which are both expressed in the heart and blood vessels, as a result of the degradation of haem.^{37,38} The oxidative degradation of haem, catalyzed by HO, results in the formation of iron (Fe^{2+}), CO and biliverdin.²⁸ Biliverdin is further converted into biologically important antioxidant bilirubin. Comparable to the formation of NO, this reaction requires O_2 and

nicotinamide adenine dinucleotide phosphate (NADPH) as cofactors. Similar to NO, CO is also thought to have important vasoregulatory effects. For instance, CO is thought to induce vasorelaxation in large vessels (e.g., aorta) by activating sGC in VSMCs, which leads to the formation of cGMP.²⁸ In smaller vessels, CO is thought to promote vasorelaxation by affecting calcium-activated potassium channels in VSMCs.³⁹ In contrast to these findings, other studies failed to demonstrate the vasodilatory effect of CO on cerebral arteries,^{40,41} or even reported on the vasoconstricting effect of CO,⁴² presumably by negatively affecting eNOS activity.^{29,43} In addition to its vasoregulatory effect, CO is also believed to have several anti-inflammatory, anti-apoptotic and pro-apoptotic effects. For instance, it was previously shown that CO may act as an anti-apoptotic agent in ECs, and, thus, prevent cell injury through Akt activation, and by affecting p38 mitogen-activated protein kinases (MAPK) signaling pathway.^{44–46} In contrast, CO can also arrest fibroblast proliferation⁴⁷ as well as VSMC proliferation.⁴⁸ Considerable evidence supports a protective role for the HO-1/CO system in several cardiovascular diseases (e.g., atherosclerosis, myocardial infarction, and hypertension). The protective effect of CO in these diseases is thought to be associated with prevention of oxidative stress-induced endothelial damage,⁴⁴ increased production of bilirubin,⁴⁹ increased stability of HIF-1 α ,⁵⁰ and improved vasorelaxation.³⁹ Given the fact that some of these diseases may share a common pathogenesis, it could be postulated that CO might also have several beneficial effects in SSs patients.

THE ROLE OF HYDROGEN SULFIDE IN VASCULAR BIOLOGY

PRODUCTION OF H₂S

Hydrogen sulfide is an evolutionary gaseous signaling molecule, and the earliest forms of life on earth depended on sulfur as an energy source and not oxygen. Only at the time that oxygen levels started to rise on earth, sulfide syntrophy largely disappeared. It wasn't until the last couple of decades that H₂S was rediscovered in mammals as being an endogenously produced gasotransmitter, and its important functions have now been increasingly recognized.⁵¹ Although high concentrations of H₂S are believed to be toxic, enhancement of its physiological effects has been proven to be beneficial in several diseases.

This endogenous gasotransmitter is enzymatically generated by four enzymes, namely cystathionine β -synthase (CBS), cystathionine γ -lyase (CSE), cysteine aminotransferase (CAT) in conjunction with 3-mercaptopyruvate sulfurtransferase (3MST).^{52–55} These H₂S producing enzymes are expressed in various vascular cell types (e.g., ECs and VSMCs). Both CBS and CSE are found in the cytosol, whereas 3MST is mainly found in the mitochondria.⁵⁶ In ECs, CSE activity, which is strictly regulated by intracellular Ca²⁺ concentrations, is the main contributor to H₂S production.⁵⁷ Both of the pyridoxal-5 phosphate-dependent enzymes (i.e., CSE and CBS) play an important role in the transsulfuration pathway.^{56,58} During this process, homocysteine is converted into cystathionine by CBS, which is then converted by CSE to L-cysteine (Figure 1). Ammonium and pyruvate are also produced during this process. However, their exact roles in cellular functions remain not well

clarified.⁵⁹ Although CSE, CBS, 3MST and CAT are essential for the formation of H_2S , it should be realized that these enzymes are also responsible for a number of other reactions that do not lead to the production of H_2S .⁵⁸ Although H_2S can also be produced through several non-enzymatic processes (e.g., through reductive chemistry of various forms of sulfur including thiosulfate, thiocystine and sulfite), these pathways are less well understood and only account for a small portion of H_2S production.⁶⁰

The concentration of H_2S in human blood was previously estimated to range from 10–100 μM ,^{61,62} however, recent studies using more precise methods reported values of only 14–15 nanomolar.^{63,64} Moreover, it should be noted that the measured H_2S concentration is strongly dependent on the age of the subject and the method used.⁶⁵

Figure 1. Schematic presentation of the production and function of hydrogen sulfide.

Hydrogen sulfide can be quickly eliminated and is initially oxidized to thiosulfate. Following this process thiosulfate is further converted into sulfite and sulfate (Figure 1). Other routes of elimination include binding to methemoglobin and cytosolic methylation.⁶⁶ Given the fact that thiosulfate can easily be converted back into H₂S, this sodium salt is often considered as an H₂S donor. Sulfate is considered an important end-product of H₂S metabolism. In humans, the majority of the circulatory inorganic sulfate is generated from the sulfur containing amino acids (SAA) methionine and cysteine, both derived from dietary protein.^{67,68} However, because of the beneficial effects of H₂S, it can also be hypothesized that higher intake of SAAs contributes to the formation of H₂S, and, thereby, beneficially influencing the cardiovascular profile and, as a result, patient survival.^{69,70} Therefore, sulfate levels could possibly also serve as an indication of H₂S production. However, in order to understand the role of this important gasotransmitter in pathophysiology of many vascular diseases (including SSc), one has to comprehend the role it plays in normal vascular biology.

VASCULAR BIOLOGY

VASCULAR TONE REGULATION

Regulation of the vascular tone is crucial in maintaining adequate blood flow and tissue oxygenation to the organs. This balance in vasodilation and vasoconstriction is strictly regulated by various factors, among which H₂S plays an important role. Hydrogen sulfide is believed to have a biphasic effect on the vascular tone, through the mediation of both vasoconstriction and vasodilation. Lower concentrations of H₂S are believed to induce vasoconstriction. This postulation is supported by the fact that reversal of the vasodilatory effect of acetylcholine and histamine, which are both NO-dependent vasodilators, was achieved after treatment with H₂S in lower concentrations (<100 μM).⁷¹ In addition, even very low concentrations of 30 μM were found to induce a strong vasoconstrictive effect. This vasoconstrictive effect is believed to be induced through the inactivation of NO, down-regulation of eNOS, increase in reactive oxygen species (ROS) production, and through the decrease of intracellular cyclic adenosine monophosphate (cAMP) in VSMCs.^{55,72} Moreover, it was previously demonstrated that the vasoconstrictive effect of H₂S was endothelial dependent in phenylephrine-precontracted rat aorta.⁷³ This experimental study demonstrated that the contractile effect of H₂S disappeared after the removal of the endothelium. Interestingly, the contractile activity of H₂S was found to be more prominent in the absence of oxygen.⁷⁴

Conversely, higher levels are believed to promote vasodilation by affecting K_{ATP} channels, Ca²⁺ channels, and the Cl⁻/HCO₃⁻ exchanger (Figure 2). In particular, H₂S leads to hyperpolarization of K_{ATP} channels in VSMCs, which reduces intracellular Ca²⁺, and leads to the relaxation of smooth muscle cells.⁷⁵ However, given the fact that glibenclamide, which is a K_{ATP} inhibitor, only partially prevented vasodilation, it has to be noted that the vasodilatory effect of H₂S is not solely exerted through its effect on the K_{ATP} channels.⁷⁶ In addition, H₂S also induces vasodilation by modulating K_v7 channels in VSMCs,⁷⁶ and by affecting K_{Ca} channels.⁷⁷ Moreover, the inhibition of cytochrome c oxidase by H₂S may

lead to a decrease in cellular ATP levels and activate K_{ATP} channels, which subsequently promotes vasodilation.^{78,79} In contrast to the vasoconstrictive effect of H_2S , vasodilation is thought to be promoted by the activation of eNOS and stimulation of NO-release.⁸⁰ In addition, it has been shown that the intermediates that form due to the NO- H_2S crosstalk, might also play an important role in this process. Hydrogen sulfide has been proven to reduce the blood pressure in various experimental models. More particularly, several experimental animal studies in mice have shown a reduction of the blood pressure after the administration of both systemic and local H_2S donors (e.g., NaHS).^{81–83} More recently, it has been reported that H_2S and NO, which are both key regulators in various physiological functions, are able to interact with each other and form intermediates with important physiological functions.⁸⁴ For instance, H_2S was found to reduce oxidized NO forms, and thereby form several intermediate products (e.g., SSNO⁻, HSNO, HNO, and HSSH) which are highly redox-sensitive.⁸⁴ Therefore, it has become increasingly clear that the interactions between NO and H_2S may have a significant effect on the regulation of the vascular tone. In support, Hosoki et al. (1997) demonstrated that H_2S can induce a stronger vasorelaxant effect in the presence of an NO donor.⁸⁵ Another study reported that HNO may induce vasodilation by activating the cGMP-dependent pathway.⁸⁶ A recent review describes these interactions between gasotransmitters in the redox system as the “redox signaling interactome”, and these interactions may lead to targets for new therapeutic interventions.⁸⁷

Figure 2. Physiological effects of intracellular hydrogen sulfide production in vascular smooth muscle cells and endothelial cells.

Cell proliferation and angiogenesis

Hydrogen sulfide was previously reported to stimulate EC proliferation, which is an essential part of the initial angiogenic response. Two independent studies reported that H_2S , in various concentration (ranging from 1-600 μM), increased proliferation of human umbilical vein endothelial cells (HUVECs) and of transformed ECs by 30-100%.^{88,89} Moreover, H_2S , at low concentrations (10-20 $\mu mol/l$) promotes EC migration and adhesion, which may further stimulate angiogenesis. Regarding the angiogenic properties of H_2S , Cai and colleagues (2007) reported that intraperitoneal administration of an H_2S donor (NaHS, 10-20 μmol per kg/day for 7 days) increased neovascularization in a mouse model.⁸⁸ However, higher concentrations of this H_2S donor in the same assay failed to demonstrate this angiogenic property. This difference might be due to the bell-shaped biological dose-response, with lower doses having a cytoprotective effect, whereas a higher dosage might have a more pronounced cytotoxic effect.^{90,91}

There are several intracellular signaling pathways which play an important role in the pro-angiogenic effect of H_2S . For instance, vascular endothelial growth factor (VEGF) plays a significant role in the angiogenesis, both in physiology and pathophysiology.⁹² Moreover, it has previously been reported that VEGF promotes H_2S synthesis, presumably through calcium/calmodulin-dependent activation of CSE.⁹³ Other important signaling pathways include the phosphatidylinositol 3-kinase-Akt-survivin pathway, the extracellular signal-regulated kinase and p38 pathways.⁹⁴ In addition, there is some evidence supporting the role of NO in this process,⁹⁵ however, its exact implication in disease must be further investigated.

Antioxidant effect of H_2S

Hydrogen sulfide, in low concentrations, is believed to have a strong antioxidant capacity, which is mainly due to its ROS inhibiting and scavenging property. However, studies on its antioxidant mechanism are limited. Evidence exists indicating that H_2S up-regulates glutathione (GSH) and increases the expression of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase.⁹⁶⁻⁹⁸ In addition, H_2S was reported to increase intracellular thioredoxin (Trx-1). This multifunctional molecule not only has ROS scavenging properties, but also plays an important role in cell proliferation,⁹⁹ apoptosis,¹⁰⁰ and gene expression.¹⁰¹ More recently, increasing evidence revealed that the transcription factor nuclear factor like 2 (Nrf2) may regulate the antioxidant capacity of H_2S , by promoting cellular antioxidant gene expression (e.g., glutamate cysteine ligase regulatory subunit, glutamate cysteine ligase catalytic subunit).¹⁰² This transcription factor can also increase the expression of glutathione reductase, which promotes the recycling of GSH and increases GSH/GSSG ratio, and, thereby, reduces oxidative stress.¹⁰³

THE POTENTIAL ROLE OF H₂S IN THE DEVELOPMENT OF SYSTEMIC SCLEROSIS-RELATED VASCULOPATHY

LINK BETWEEN H₂S AND VASCULAR INJURY

The vascular endothelium is considered to be a major target for pathological processes in SSc.⁷ Damage to the endothelium is characterized by EC apoptosis, vascular endothelial permeability and impairment of cell–cell adhesion, which may lead to alterations in EC signal transduction.¹⁰⁴ Collectively, this may lead to endothelial dysfunction, subsequently causing an impaired relaxation of the vessels. Patients often present with RP as the earliest manifestation of this endothelial dysfunction.¹⁰⁵

Homocysteine (Hcy), which is converted into H₂S through the transsulfuration pathway, is considered to be a potential mediator in the initiation of endothelial dysfunction in SSc. It was previously reported that increased levels of Hcy are associated with a higher risk of several cardiovascular diseases including atherosclerosis, renal and cerebrovascular diseases.¹⁰⁶ Interestingly, Hcy is also found to be elevated in SSc patients.^{107–109} The exact reason for this elevation is unknown. Although the causal relationship between genetic abnormalities and the development of SSc has never been demonstrated, it could be hypothesized that the elevated levels of Hcy may be caused by a defect in the transsulfuration pathway. For instance, Hankey et al. (1999) stated that the most common enzyme defect associated with moderately raised homocysteine level is a point mutation in the coding region of the gene for methylenetetrahydrofolate reductase (MTHFR).¹¹⁰ Szamosi et al. (2009) aimed to investigate the prevalence of MTHFR gene polymorphism in SSc patients.¹¹¹ Although they did not demonstrate any differences in Hcy level and MTHFR genotypes, as compared to healthy controls, they did observe significantly higher levels of Hcy in patients with macroangiopathy. Therefore, they concluded that Hcy levels might be associated with the occurrence/development of SSc-related vasculopathy.

Moreover, elevated levels of Hcy can also be caused by a reduced vitamin intake or vitamin deficiency (e.g., folate, vitamin B12, vitamin B6).¹¹² This defect could lead to a decreased production of H₂S. A previously conducted experimental animal study in CBS deficient mice showed that *cbs*^{+/-} mice had a normal mean lifespan, and only developed a mild increase in plasma Hcy levels.¹¹³ Moreover, other studies reported that *cbs*^{+/-} and *cbs*^{-/-} mice demonstrate endothelial dysfunction in both large and smaller vessels.^{114,115} Hcy is believed to induce an oxidative milieu by stimulating the production of ROS (by the up-regulation of nicotinamide adenine dinucleotide phosphate),¹¹⁶ and inhibiting antioxidant enzymes (including intracellular glutathione peroxidase).^{117–119} These superoxide anions may react with NO, which leads to the formation of peroxynitrite, and a decreased bioavailability of NO, which may further impair the endothelial function.¹²⁰ The endothelial dysfunction in experimental animal studies can be largely attributed to the increase in oxidative stress and decrease of NO bioavailability. In addition, higher levels of Hcy may also lead to a decrease in H₂S production, which is believed to be caused by a decrease in CSE activity. This decrease in CSE activity was previously demonstrated in an experimental animal study in which homocysteine at higher levels reduced CSE activity in rat liver.¹²¹

Moreover, Chang et al. (2008) reported that although cardiac CSE mRNA expression was up-regulated in the HHcy rats, CSE activity was inhibited by 50%.¹²² Furthermore, the administration of H_2S in this animal study slightly lowered Hcy level, decreased myocardial lipid peroxidation and reduced ROS generation. The decrease in CSE activity may further increase the susceptibility to endothelial injury in SSc patients. The importance of Hcy in the development and perpetuation of endothelial injury is also supported by Caramaschi et al. (2007). They not only observed a higher level of Hcy in SSc patients, as compared to healthy controls, but also demonstrated that plasma concentration of Hcy is strongly associated with nailfold videocapillaroscopy (NVC) patterns, which is commonly used to assess SSc specific microvascular lesions (Figure 3).^{107,123} This relationship was found to be irrespective of age, RP, SSc duration, the severity of skin involvement and folate levels. These findings are further supported by the observations of Motegi et al. (2014) stating that the plasma concentration of Hcy increases with the progression of nailfold abnormalities.¹²⁴ These observations may suggest that even mildly increased levels of Hcy may cause vascular damage in SSc through the direct effect of Hcy on the endothelium, and its inhibitory effect on H_2S production. However, future studies are needed in order to clarify the exact role of Hcy in the occurrence of SSc-related vasculopathy.

It has to be noted that the level of cysteine in healthy individuals are usually higher than the level of homocysteine. Therefore, it could be debated whether the contribution of homocysteine in the production of H_2S is of great importance, in which slight deviations of the norm would lead to an impaired H_2S production. However, from a biochemical perspective, during hyperhomocysteinemia, homocysteine may compete with cysteine to bind to CSE, and, therefore, may indeed decrease H_2S production.¹²⁵ Although there is no direct evidence indicating homocysteine induced reduction of CSE activity in SSc patients, a modified CSE activity, as proposed above, may indeed attenuate H_2S generation.

Figure 3. Nail-fold capillaroscopy patterns of a healthy subject (A) and a subject with SSc (B).

The recurrent vasospastic events, known as RP, cause repetitive and prolonged ischemia followed by reperfusion (I/R injury), and may lead to substantial tissue hypoxia.¹²⁶ Some evidence exists indicating that hypoxia can up-regulate the expression of CSE, which may partly protect the cells from hypoxia-induced injury.¹²⁷ However, other reports

have also shown decreased CSE expression, as a result of suppressed expression of the transcription factor specificity protein 1 (Sp1).¹²⁸ These conflicting observations underline the complexity of the role played by H_2S in hypoxic conditions. Hydrogen sulfide is also believed to down-regulate hypoxia-inducible factor 1- α (HIF-1 α) protein levels in cultured cells under hypoxic conditions.¹²⁹ This inhibitory effect was achieved with 100 μ M of sodium hydrosulfide (NaHS) or 10 μ M of sodium sulfide (Na_2S).

Vascular injury in SSc may also be the result of impaired angiogenesis. Increasing evidence suggests that an imbalance of pro-angiogenic and anti-angiogenic mediators may be responsible for the impaired angiogenesis.¹³⁰ A previously conducted study demonstrated that ECs isolated from SSc patients show an impaired response to VEGF.¹³¹ Hydrogen sulfide is believed to play a significant role in the essential components of the angiogenic response, including proliferation and migration of endothelial cells. Two studies reported that H_2S leads to an increase of 20% in the proliferation of transformed endothelial cells, and 100% increase in HUVEC cells, in addition to a 30% increase in mitigation in transformed endothelial cells and a six-fold increase in HUVEC cells.^{89,94} The pathways involved in these processes have been studied, and H_2S was found to activate PI-3K/Akt axis, enhance the phosphorylation of MAPK pathway and induce hsp27 phosphorylation.^{88,89} Furthermore, it is thought that K_{ATP} -channel opening, more particularly K^+ efflux, can promote EC motility.⁸⁹ In addition, it was previously demonstrated that VEGF increases the production of H_2S , which may subsequently lead to VEGF-induced and H_2S -induced EC migration. A current hypothesis is that both VEGF and H_2S -induced EC migration involve the same downstream pathways, and this assumption is supported by Papapetropoulos et al. (2009) who reported that “VEGF-induced angiogenesis was suppressed in aortic rings of CSE-deficient mice”.⁸⁹

LINK BETWEEN H_2S AND INFLAMMATION

Endogenously synthesized H_2S is believed to be an important mediator of inflammation,¹³² and, therefore, may play an important role in the pathogenesis of SSc-related vasculopathy. Current literature has provided some conflicting results on the exact role of H_2S in the inflammatory process. For instance, H_2S is thought to have both pro-inflammatory and anti-inflammatory effects. This is presumably due to the biphasic effect of H_2S , with low concentrations inhibiting inflammation and higher concentrations inducing inflammation. The pro-inflammatory effect is thought to be induced by the activation of adenosine triphosphate (ATP)-sensitive K^+ channels, and this was previously observed in mice with septic shock.¹³³ The administration of a CSE inhibitor, which decreases the H_2S level, was shown to increase survival in this experimental study.¹³³ Moreover, in an experimental animal study, Zanardo et al. (2006) demonstrated that H_2S donors inhibit leukocyte adherence during vascular inflammation, which further supports the anti-inflammatory effects of H_2S .¹³⁴ In another experimental animal study the administration of H_2S donors, such as NaSH, was found to induce inflammation through the up-regulation of nuclear factor- κ B (NF- κ B) and extracellular-signal-regulated kinase (ERK) 1/2 signaling.¹³⁵ Furthermore, Rinaldi et al. (2006) found that H_2S prevented apoptosis of human

granulocytes via the inhibition of p38 mitogen-activated protein kinases and caspase 3.¹³⁶ Conversely, H_2S is also thought to have several anti-inflammatory effects including free radical scavenging properties (e.g., peroxynitrite), inhibition of leukocyte adherence to the endothelium,¹³⁴ and H_2S is believed to reduce the leukocyte infiltration.^{137,138}

LINK BETWEEN H_2S AND FIBROSIS

Hyperhomocysteinemia might be a contributing factor in the increased production of collagen in SSc patients.¹³⁹ Hyperhomocysteinemia-induced oxidative stress is thought to be a key player in this process. During this process matrix metallo-proteinases (MMPs) are believed to be activated, which subsequently cause disturbances in the metabolism of extracellular matrix (ECM). Moreover, Hcy is believed to lead to vascular stiffness by regulating the elastin/collagen ratio.¹⁴⁰ As increased levels of Hcy are known to decrease the CSE activity, it could be postulated that hyperhomocysteinemia leads to a decreased production of H_2S , which may further sustain the pro-fibrotic state.¹²² Conversely, hydrogen sulfide itself is believed to have several anti-fibrotic effects. For instance, H_2S is thought to inhibit myofibroblast activation, which is a key event in the pathogenesis of fibrosis in SSc.¹⁴¹ In addition, the anti-inflammatory effect of H_2S (e.g., decreasing the expression of inflammatory cytokines) is also thought to block/reduce the disease-related pro-fibrotic state. Wang and colleagues demonstrated that NaHS, which is an H_2S donor, inhibits the fibroblast differentiation and attenuates the protein expressions of TGF- β 1.¹⁴² In support, several others have concluded “that organ fibrosis might be associated with H_2S deficiency”.^{143,144} The blockage of TGF- β 1-induced fibroblast differentiation is presumably achieved through the inhibition of the phosphorylation of Smad3 and mitogen-activated protein kinases.¹⁴⁵ Furthermore, ROS are also thought to play a crucial role in the formation of fibrosis in SSc patients.¹⁴⁶ Hydrogen sulfide has been demonstrated to have ROS-scavenging properties and, thereby, eliminates the fibrosis-inducing properties of ROS. Moreover, it was previously shown that the administration of an H_2S donor can lead to the increased expression of antioxidant enzymes (e.g., manganese superoxide dismutase, catalase and copper-zinc superoxide dismutase), which reduces the oxidative milieu.¹⁴⁷ These findings are further supporting the suggestion that H_2S might play a pivotal role in the pathogenesis of SSc-related vasculopathy.

HYDROGEN SULFIDE AS A THERAPEUTIC OPTION

Exogenous alterations of H_2S levels can be done in several ways, including, but not limited to, treatment with sulfide-sodium salts, exposure to gaseous H_2S , slow-releasing H_2S donors, and by improving the endogenously produced H_2S (e.g., up-regulating the expression of H_2S -producing enzymes).¹⁴⁸ It wasn't until the last couple of decades that the perception of H_2S shifted and its important functions were recognized. Following this shift in perception, multiple studies have demonstrated the cytoprotective effects of H_2S .^{137,138} In particular, some have demonstrated that H_2S , in relatively low concentrations, can up-regulate endogenous antioxidant systems, increase the production of cAMP, and can up-regulate anti-inflammatory and cytoprotective genes.¹⁴⁹ In contrast to lower

concentration, a higher concentration of H_2S is believed to be cytotoxic, which is likely due to its ability to inhibit cytochrome c oxidase. The inhibition of cytochrome c oxidase in complex V, leads to a decrease in ATP production, and in the presence of oxygen may lead to the formation of ROS.¹⁵⁰ Moreover, the generation of ROS, glutathione depletion, and the induction of mitochondrial cell death pathways may also enhance or even induce an oxidative milieu.¹⁵¹ Although studies in SSc patients are lacking, several experimental animal and human studies in other vascular conditions have clearly demonstrated the beneficial effect of H_2S treatment.^{75,152–154}

HYDROGEN SULFIDE IN EXPERIMENTAL ANIMAL STUDIES

Sulfide salts (e.g., Na_2S and NaHS) have previously been used in numerous studies, and demonstrated to have protective effects in a variety of diseases including I/R-related diseases.¹⁵⁵ For instance, Zhang et al. (2013) demonstrated that preconditioning with NaHS reduced the I/R-induced hepatic injury in mice. They stated that the protective effect was presumably achieved by the inhibition of mitochondrial permeability transition pore (mPTP) opening.¹⁵⁶ Johansen et al. (2006) were the first to demonstrate the cytoprotective effect of H_2S following myocardial I/R injury.¹⁵⁷ They reported that NaHS treatment (1 μM) 10 min prior to reperfusion led to a reduction of the infarct size of 20%. Following their results, several others reported supporting results. For instance, in a recently conducted study, researchers found that injection with Na_2S (compared to vehicle) prior to the ischemia limited the extent of myocardial damage following I/R.¹⁵⁸ Moreover, they demonstrated that this sulfide salt reduced oxidative stress and apoptosis, increased the nuclear accumulation of Nrf2, increased the expression of Trx1 and HO-1 and activated a PKC ϵ -p44/42-STAT-3 pro-survival signaling pathway.¹⁵⁸ Comparable results were found when NaHS was injected 1 day prior to the MI.¹⁵⁹ NaHS not only reduced the myocardial infarct size, but also prevented apoptosis of cardiomyocyte. However, NaSH administered 5 days prior to MI had no effect.

It was previously proposed that H_2S may also have beneficial effects in renal IR injury. A previously conducted study demonstrated that topical treatment of NaHS on the kidneys 15 minutes prior to ischemia significantly increased the kidney function.¹⁶⁰ Another study reported that during I/R, the renal injury is caused by a reduction in CBS activity.¹⁶¹ This finding may suggest that H_2S may be hampered during I/R injury. Furthermore, subtoxic concentrations of H_2S can induce a hibernation-like state in mice.^{162,163} It was previously reported that mice, treated with gaseous hydrogen sulfide, showed a reduction of >90% in metabolic rate, and a decline in core body temperature to near ambient temperature. Moreover, H_2S is also known to modulate the blood pressure, and, therefore, may serve as an add-on treatment option in hypertension. In various experimental mice models, H_2S was found to reduce the arterial pressure after systemic treatment with NaHS,^{164,165} or after local treatment.¹⁶⁶ These findings are supported by Huang et al. (2015), they stated that NaHS reduced systolic pressure in Dahl rats with high salt-induced hypertension.¹⁶⁷

It has become increasingly clear that H_2S is also abundantly produced by gut microbiota through sulfate-reducing bacteria. Sulfate-reducing bacteria are a non-enzymatic source of H_2S , and several bacteria (e.g., *E. coli*, *Salmonella*, and *Enterobacter*) are known to have the ability to produce H_2S .¹⁶⁸ It was previously shown that antibiotic treatment aimed at reducing the intestinal microbiota, resulted in a significant reduction of thiosulfate levels.¹⁶⁹ This finding sheds light on sulfate-reducing bacteria as a therapeutic target in various vascular diseases.¹⁶⁸

The only SSc-resembling experimental animal study was conducted by Wang and colleagues (2016) who investigated the effect of NaHS (56 and 112 $\mu\text{g/kg}$), in an experimental scleroderma-like animal study in mice.¹⁴² They demonstrated that the plasma H_2S levels were decreased in mice after injection with bleomycin. Moreover, they stated that H_2S exerts an anti-inflammatory effect by the reduction of macrophage recruitment in lung tissues. In addition, they also stated that NaHS attenuates the protein expressions of TGF- $\beta 1$, indicating that H_2S also inhibits the fibroblast differentiation and extracellular matrix production. In support, several others have concluded “that organ fibrosis might be associated with H_2S deficiency”.^{143,144} These findings are further supporting the suggestion that H_2S can be seen as a promising treatment option in SSc. The hypothetical effects of H_2S treatment in SSc can be seen in figure 4.

Figure 4. Hypothetical inhibitory and promoting effects of hydrogen sulfide treatment in the vasculature of SSc patients.

HYDROGEN SULFIDE IN HUMAN STUDIES

Despite the fact that several experimental animal studies clearly demonstrated the beneficial effects of treatment with sulfide salts, large studies in humans are still lacking. Thereby, the majority of H_2S -releasing derivatives are not yet used in clinical settings. However, sodium thiosulfate (STS) is the exception, given the fact that this sodium salt is already used in various diseases. For instance, STS is a well-known effective antidote used in cyanide intoxication. Moreover, STS was proven to be beneficial as a treatment of cisplatin-induced hearing loss. For instance, an open-label, randomized trial demonstrated that hearing loss was significantly lower in children treated with STS, as compared to the control group.¹⁷⁰ Importantly, they also reported that the use of STS was not associated with the occurrence of serious adverse events. Sodium thiosulfate is also used in calciphylaxis in patients with end-stage renal disease.¹⁷¹ Calciphylaxis is a rare disorder characterized by calcification of the tunica media, and is often associated with high mortality rates. Calciphylaxis is a dreaded complication and often leads to ulceration of the extremities and contributes to the development of peripheral arterial disease.¹⁷² This dreaded complication can also occur in SSc and remains virtually untreatable. Several studies have shown that STS has beneficial effects on cutaneous lesions, with a potentially long-lasting effect (11 months-3 years).¹⁷³ In a multi-centric study of 27 calciphylaxis patients treated with STS, 70% of these patients had a complete or partial response.¹⁷⁴ Although these effects are presumably mediated through different pathways as compared to the effects as hypothesized in SSc, these promising results indicate that STS can be safely used in the clinical setting. In our hospital, STS is already in a Phase III clinical trial designed to evaluate the effect of sodium thiosulfate compared with placebo treatment, in adjunction to optimal reperfusion therapy for acute MI, on left ventricular ejection fraction.

CONCLUSION

Vasculopathy is the hallmark of SSc and often leads to severe disability and morbidity. To date, SSc is still associated with increased mortality rates given the fact that effective disease-modifying treatment options are currently unavailable. Hydrogen sulfide donors have previously been proven to have beneficial effects in a variety of diseases. These effects range from ROS scavenging, anti-fibrotic, pro-angiogenic to vasoactive properties. Although the pathways involved in H_2S production have not been clearly investigated in SSc, current data may suggest that the readily available H_2S -donor STS may have a great therapeutic potential. Moreover, given the abundant experience in other diseases, with minimal adverse events reported, this drug could probably be safely administered in SSc. A current hypothesis is that early treatment of SSc with STS may alter or even arrest disease progression. Although future studies are needed in order to investigate this hypothesis, the promising effects of H_2S -based therapeutic targets may encourage further study in SSc.

REFERENCES

1. Leroy EC, Black C, Fleischmajer R, et al. Scleroderma (Systemic Sclerosis): Classification, Subsets and Pathogenesis. *J Rheumatol*. 1988;15(2):202-205.
2. Denton CP, Khanna D. Systemic sclerosis. *Lancet*. 2017;390(10103):1685-1699. doi:10.1016/S0140-6736(17)30933-9
3. Almeida C, Almeida I, Vasconcelos C. Quality of life in systemic sclerosis. *Autoimmun Rev*. 2015;14(12):1087-1096. doi:10.1016/j.autrev.2015.07.012
4. Nikpour M, Stevens WM, Herrick AL, Proudman SM. Epidemiology of systemic sclerosis. *Best Pract Res Clin Rheumatol*. 2010;24(6):857-869. doi:10.1016/j.berh.2010.10.007
5. Ranque B, Mouthon L. Geoepidemiology of systemic sclerosis. *Autoimmun Rev*. 2010;9(5):A311-A318. doi:10.1016/j.autrev.2009.11.003
6. Chiffot H, Fautrel B, Sordet C, Chatelus E, Sibilia J. Incidence and Prevalence of Systemic Sclerosis: A Systematic Literature Review. *Semin Arthritis Rheum*. 2008;37(4):223-235. doi:10.1016/j.semarthrit.2007.05.003
7. Mostmans Y, Cutolo M, Giddelo C, et al. The role of endothelial cells in the vasculopathy of systemic sclerosis: A systematic review. *Autoimmun Rev*. 2017;16(8):774-786. doi:10.1016/j.autrev.2017.05.024
8. Abdulle AE, van Roon AM, Smit AJ, et al. Rapid free thiol rebound is a physiological response following cold-induced vasoconstriction in healthy humans, primary Raynaud and systemic sclerosis. *Physiol Rep*. 2019;(6):e14017. doi:10.14814/phy2.14017
9. Herrick AL. The pathogenesis, diagnosis and treatment of Raynaud phenomenon. *Nat Rev Rheumatol*. 2012;8(8):469-479. doi:10.1038/nrrheum.2012.96
10. Denton CP, Khanna D. Systemic sclerosis. *Lancet*. 2017;6736(17):1-15. doi:10.1016/S0140-6736(17)30933-9
11. Polhemus DJ, Lefer DJ. Emergence of hydrogen sulfide as an endogenous gaseous signaling molecule in cardiovascular disease. *Circ Res*. 2014;114(4):730-737. doi:10.1161/CIRCRESAHA.114.300505
12. Mann BE, Motterlini R. CO and NO in medicine. *Chem Commun*. 2007;(41):4197-4208. doi:10.1039/b704873d
13. Wang R. Two's company, three's a crowd: can H₂S be the third endogenous gaseous transmitter? *FASEB J*. 2002;16(13):1792-1798. doi:10.1096/fj.02-0211hyp
14. Matucci Cerinic M, Kahaleh MB. Beauty and the beast. The nitric oxide paradox in systemic sclerosis. *Rheumatology (Oxford)*. 2002;41(8):843-847.
15. Cotton SA, Herrick AL, Jayson MI V, Freemont AJ. Endothelial expression of nitric oxide synthases and nitrotyrosine in systemic sclerosis skin. *J Pathol*. 1999;189:273-278. doi:10.1002/(SICI)1096-9896(199910)189:2<273::AID-PATH413>3.0.CO;2-4
16. Allano Y, Borderie D, Hilliquin P, et al. Low levels of nitric oxide (NO) in systemic sclerosis: inducible NO synthase production is decreased in cultured peripheral blood monocyte/macrophage cells. *Rheumatology (Oxford)*. 2001;40(10):1089-1096. <http://www.ncbi.nlm.nih.gov/pubmed/11600736>.
17. Dooley A, Gao B, Bradley N, et al. Abnormal nitric oxide metabolism in systemic sclerosis: Increased levels of nitrated proteins and asymmetric dimethylarginine. *Rheumatology*. 2006;45(6):676-684. doi:10.1093/rheumatology/kei276
18. Malerba M, Radaeli A, Ragnoli B, et al. Exhaled nitric oxide levels in systemic sclerosis with and without pulmonary involvement. *Chest*. 2007;132(2):575-580. doi:10.1378/chest.06-2929
19. Takagi K, Kawaguchi Y, Hara M, Sugiura T, Harigai M, Kamatani N. Serum nitric oxide (NO) levels in systemic sclerosis patients: Correlation between NO levels and clinical features. *Clin Exp Immunol*. 2003;134(3):538-544. doi:10.1111/j.1365-2249.2003.02320.x

20. Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. *Prog Cardiovasc Dis*. 1995;38(2):87-104. doi:10.1016/S0033-0620(05)80001-5
21. Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*. 1988;333(6174):664-666. doi:10.1038/333664a0
22. Zhao Y, Vanhoutte PM, Leung SWS. Vascular nitric oxide: Beyond eNOS. *J Pharmacol Sci*. 2015;129(2):83-94. doi:10.1016/j.jphs.2015.09.002
23. Kröncke KD, Fehsel K, Kolb-Bachofen V. Inducible nitric oxide synthase in human diseases. *Clin Exp Immunol*. 1998;113(2):147-156. doi:10.1046/j.1365-2249.1998.00648.x
24. Marietta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS. Macrophage Oxidation of L-Arginine to Nitrite and Nitrate: Nitric Oxide Is an Intermediate. *Biochemistry*. 1988;27(24):8706-8711. doi:10.1021/bi00424a003
25. Yui Y, Hattori R, Kosuga K, et al. Calmodulin-independent nitric oxide synthase from rat polymorphonuclear neutrophils. *J Biol Chem*. 1991;266(6):3369-3371.
26. Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci*. 1977;74(8):3203-3207. doi:10.1073/pnas.74.8.3203
27. Carvajal JA, Germain AM, Huidobro-Toro JP, Weiner CP. Molecular mechanism of cGMP-mediated smooth muscle relaxation. *J Cell Physiol*. 2000;184(3):409-420. doi:10.1002/1097-4652(200009)184:3<409::AID-JCP16>3.0.CO;2-K
28. Sammut IA, Foresti R, Clark JE, et al. Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of haeme oxygenase-1. *Br J Pharmacol*. 1998;125(7):1437-1444. doi:10.1038/sj.bjp.0702212
29. Thorup C, Jones CL, Gross SS, Moore LC, Goligorsky MS. Carbon monoxide induces vasodilation and nitric oxide release but suppresses endothelial NOS. *Am J Physiol Physiol*. 1999;277(6):F882-F889. doi:10.1152/ajprenal.1999.277.6.F882
30. Freedman RR, Girgis R, Mayes MD. Endothelial and adrenergic dysfunction in Raynaud's phenomenon and scleroderma. *J Rheumatol*. 1999;26(11):2386-2388.
31. Romero LI, Zhang DN, Cooke JP, et al. Differential expression of nitric oxide by dermal microvascular endothelial cells from patients with scleroderma. *Vasc Med*. 2000;5(3):147-158.
32. Nara K, Konno D, Uchida J, Kiuchi Y, Oguchi K. Protective effect of nitric oxide against iron-induced neuronal damage. *J Neural Transm*. 1999;106:835-848. doi:10.1007/s007020050204
33. O'Donnell VB, Freeman BA. Interactions between nitric oxide and lipid oxidation pathways: Implications for vascular disease. *Circ Res*. 2001;88:12-21. doi:10.1161/01.RES.88.1.12
34. Lind M, Hayes A, Caprnda M, et al. Inducible nitric oxide synthase: Good or bad? *Biomed Pharmacother*. 2017;93(2017):370-375. doi:10.1016/j.biopha.2017.06.036
35. Yamamoto T, Katayama I, Nishioka K. Nitric oxide production and inducible nitric oxide synthase expression in systemic sclerosis. *J Rheumatol*. 1998;25(2):314-317.
36. Sud A, Khullar M, Wanchu A, Bamberg P. Increased nitric oxide production in patients with systemic sclerosis. *Nitric Oxide - Biol Chem*. 2000;4(6):615-619. doi:10.1006/niox.2000.0309
37. Ewing JF, Maines MD. Rapid induction of heme oxygenase 1 mRNA and protein by hyperthermia in rat brain: heme oxygenase 2 is not a heat shock protein. *Proc Natl Acad Sci U S A*. 1991;88(12):5364-5368. doi:10.1073/pnas.88.12.5364
38. Schipper HM. Heme oxygenase-1: Transducer of pathological brain iron sequestration under oxidative stress. *Ann N Y Acad Sci*. 2004;1012:84-93. doi:10.1196/annals.1306.007
39. Wang R, Wu L. The chemical modification of K(Ca) channels by carbon monoxide in vascular smooth muscle cells. *J Biol Chem*. 1997;272:32804-32809. doi:10.1074/jbc.272.13.8222

40. Ndisang J, Tabien H, Wang R. Carbon monoxide and hypertension. *J Hypertens*. 2004;22:1057-1074.
41. Andresen J, Shafi N, Durante W, Bryan RJ. The effect of carbon monoxide and heme oxygenase inhibitors in cerebral vessels of rats and mice. *Am J Physiol Hear Circ Physiol*. 2006;291(1):H223-30. doi:10.1152/ajpheart.00058.
42. Johnson FK, Johnson R a. Carbon monoxide promotes endothelium-dependent constriction of isolated gracilis muscle arterioles. *Am J Physiol Regul Integr Comp Physiol*. 2003;285(3):R536-41. doi:10.1152/ajpregu.00624.2002
43. Foresti R, Hammad J, Clark JE, et al. Vasoactive properties of CORM-3, a novel water-soluble carbon monoxide-releasing molecule. *Br J Pharmacol*. 2004;142(3):453-460. doi:10.1038/sj.bjp.0705825
44. Brouard S, Otterbein LE, Anrather J, et al. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *J Exp Med*. 2000;192:1015-1026. doi:10.1084/jem.192.7.1015
45. Kim HS, Loughran PA, Rao J, Billiar TR, Zuckerbraun BS. Carbon monoxide activates NF- B via ROS generation and Akt pathways to protect against cell death of hepatocytes. *AJP Gastrointest Liver Physiol*. 2008;295. doi:10.1152/ajpgi.00105.2007
46. Kim KM, Pae HO, Zheng M, Park R, Kim YM, Chung HT. Carbon monoxide induces heme oxygenase-1 via activation of protein kinase R-like endoplasmic reticulum kinase and inhibits endothelial cell apoptosis triggered by endoplasmic reticulum stress. *Circ Res*. 2007;(101):919-927. doi:10.1161/CIRCRESAHA.107.154781
47. Zhou Z, Song R, Fattman CL, et al. Carbon monoxide suppresses bleomycin-induced lung fibrosis. *Am J Pathol*. 2005;166:27-37. doi:10.1016/S0002-9440(10)62229-8
48. Peyton KJ, Reyna S V., Chapman GB, et al. Heme oxygenase-1-derived carbon monoxide is an autocrine inhibitor of vascular smooth muscle cell growth. *Blood*. 2002;99:4443-4448. doi:10.1182/blood.V99.12.4443
49. Clark JE, Foresti R, Sarathchandra P, Kaur H, Green CJ, Motterlini R. Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction. *Am J Physiol Heart Circ Physiol*. 2000;278(2):H643-651. doi:10.1090/S0025-5718-02-01482-5
50. Choi YK, Kim CK, Lee H, et al. Carbon monoxide promotes VEGF expression by increasing HIF-1 α protein level via two distinct mechanisms, translational activation and stabilization of HIF-1 α protein. *J Biol Chem*. 2010;285:32116-32125. doi:10.1074/jbc.M110.131284
51. Kimura Y, Goto Y-I, Kimura H. Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. *Antioxid Redox Signal*. 2010;12(1):1-13. doi:10.1089/ars.2008.2282
52. Li L, Rose P, Moore PK. Hydrogen Sulfide and Cell Signaling. *Annu Rev Pharmacol Toxicol*. 2011;51(1):169-187. doi:10.1146/annurev-pharmtox-010510-100505
53. Wallace JL, Wang R. Hydrogen sulfide-based therapeutics: Exploiting a unique but ubiquitous gasotransmitter. *Nat Rev Drug Discov*. 2015;14(5):329-345. doi:10.1038/nrd4433
54. Hancock JT, Whiteman M. Hydrogen sulfide and cell signaling: Team player or referee? *Plant Physiol Biochem*. 2014;78:37-42. doi:10.1016/j.plaphy.2014.02.012
55. Liu Y-H, Lu M, Hu L-F, Wong PT-H, Webb GD, Bian J-S. Hydrogen Sulfide in the Mammalian Cardiovascular System. *Antioxid Redox Signal*. 2012;17(1):141-185. doi:10.1089/ars.2011.4005
56. Kimura H. Production and Physiological Effects of Hydrogen Sulfide. *Antioxid Redox Signal*. 2014;20(5):783-793. doi:10.1089/ars.2013.5309
57. Mikami Y, Shibuya N, Ogasawara Y, Kimura H. Hydrogen sulfide is produced by cystathionine γ -lyase at the steady-state low intracellular Ca²⁺ concentrations. *Biochem Biophys Res Commun*. 2013. doi:10.1016/j.bbrc.2013.01.010
58. Kabil O, Banerjee R. Enzymology of H₂S biogenesis, decay and signaling. *Antioxid Redox Signal*. 2014;20(5):770-782. doi:10.1089/ars.2013.5339

59. Wang R. The Gasotransmitter Role of Hydrogen Sulfide. *Antioxid Redox Signal*. 2003;5(4):493-501. doi:10.1089/152308603768295249
60. Kolluru GK, Shen X, Kevil CG. A tale of two gases: NO and H₂S, foes or friends for life? *Redox Biol*. 2013;1(1):313-318. doi:10.1016/j.redox.2013.05.001
61. Richardson CJ, Magee EAM, Cummings JH. A new method for the determination of sulphide in gastrointestinal contents and whole blood by microdistillation and ion chromatography. *Clin Chim Acta*. 2000;(293):115-125. doi:10.1016/S0009-8981(99)00245-4
62. Beltowski J. Hydrogen sulfide in pharmacology and medicine - An update. *Pharmacol Reports*. 2015;67(2015):647-658. doi:10.1016/j.pharep.2015.01.005
63. Shen X, Peter EA, Bir S, Wang R, Kevil CG. Analytical measurement of discrete hydrogen sulfide pools in biological specimens. *Free Radic Biol Med*. 2012;52(2012):2276-2283. doi:10.1016/j.freeradbiomed.2012.04.007
64. Furne J, Saeed A, Levitt MD. Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. *AJP Regul Integr Comp Physiol*. 2008;295:R1479-R1485. doi:10.1152/ajpregu.90566.2008
65. Whiteman M, Moore PK. Hydrogen sulfide and the vasculature: A novel vasculoprotective entity and regulator of nitric oxide bioavailability? *J Cell Mol Med*. 2009;13:488-507. doi:10.1111/j.1582-4934.2009.00645.x
66. Wu D, Wang J, Li H, Xue M, Ji A, Li Y. Role of hydrogen sulfide in ischemia-reperfusion injury. *Oxid Med Cell Longev*. 2015;2015. doi:10.1155/2015/186908
67. Sabry ZI, Shadarevian SB, Cowan JW, Campbell JA. Relationship of dietary intake of sulphur amino-acids to urinary excretion of inorganic sulphate in man. *Nature*. 1965;206(987):931-933. doi:10.1038/206931b0
68. Houterman S, Van Faassen A, Ocke MC, et al. Is urinary sulfate a biomarker for the intake of animal protein and meat? *Cancer Lett*. 1997;114(1997):295-296. doi:10.1016/S0304-3835(97)04684-3
69. van den Berg E, Pasch A, Westendorp WH, et al. Urinary Sulfur Metabolites Associate with a Favorable Cardiovascular Risk Profile and Survival Benefit in Renal Transplant Recipients. *J Am Soc Nephrol*. 2014;25(6):1303-1312. doi:10.1681/ASN.2013050497
70. van den Born JC, Frenay A-RS, Koning AM, et al. Urinary Excretion of Sulfur Metabolites and Risk of Cardiovascular Events and All-Cause Mortality in the General Population. *Antioxid Redox Signal*. 2018;00(00):ars.2017.7040. doi:10.1089/ars.2017.7040
71. Ali MY, Ping CY, Mok Y-Y, et al. Regulation of vascular nitric oxide in vitro and in vivo; a new role for endogenous hydrogen sulphide? *Br J Pharmacol*. 2006;149(6):625-634. doi:10.1038/sj.bjp.0706906
72. Lim JJ, Liu Y-H, Khin ESW, Bian J-S. Vasoconstrictive effect of hydrogen sulfide involves downregulation of cAMP in vascular smooth muscle cells. *Am J Physiol Cell Physiol*. 2008;295(5):C1261-C1270. doi:10.1152/ajpcell.00195.2008
73. Kubo S, Kurokawa Y, Doe I, Masuko T, Sekiguchi F, Kawabata A. Hydrogen sulfide inhibits activity of three isoforms of recombinant nitric oxide synthase. *Toxicology*. 2007;241:92-97. doi:10.1016/j.tox.2007.08.087
74. Kiss L, Deitch EA, Szabó C. Hydrogen sulfide decreases adenosine triphosphate levels in aortic rings and leads to vasorelaxation via metabolic inhibition. *Life Sci*. 2008;83:589-594. doi:10.1016/j.lfs.2008.08.006
75. Holwerda KM, Karumanchi SA, Lely AT. Hydrogen sulfide: Role in vascular physiology and pathology. *Curr Opin Nephrol Hypertens*. 2015. doi:10.1097/MNH.0000000000000096
76. Martelli A, Testai L, Breschi MC, et al. Vasorelaxation by hydrogen sulphide involves activation of Kv7 potassium channels. *Pharmacol Res*. 2013;70(1):27-34. doi:10.1016/j.phrs.2012.12.005

77. Jackson-Weaver O, Osmond JM, Riddle MA, et al. Hydrogen sulfide dilates rat mesenteric arteries by activating endothelial large-conductance Ca^{2+} -activated K^{+} channels and smooth muscle Ca^{2+} sparks. *AJP Hear Circ Physiol*. 2013;304:H1446-H1454. doi:10.1152/ajpheart.00506.2012
78. Nicholls P, Kim J. Sulphide as an inhibitor and electron donor for the cytochrome c oxidase system. *Can J Biochem*. 1982;60(6):613-623. doi:10.1139/o82-076
79. Cooper CE, Brown GC. The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: Chemical mechanism and physiological significance. *J Bioenerg Biomembr*. 2008;40:533-539. doi:10.1007/s10863-008-9166-6
80. Dongó E, Beliczai-Marosi G, Dybvig AS, Kiss L. The mechanism of action and role of hydrogen sulfide in the control of vascular tone. *Nitric Oxide - Biol Chem*. 2018;81:75-87. doi:10.1016/j.niox.2017.10.010
81. Ahmad FUD, Sattar MA, Rathore HA, et al. Hydrogen sulphide and tempol treatments improve the blood pressure and renal excretory responses in spontaneously hypertensive rats. *Ren Fail*. 2014;(36):598-605. doi:10.3109/0886022X.2014.882218
82. Zhao X, Zhang L-K, Zhang C-Y, et al. Regulatory effect of hydrogen sulfide on vascular collagen content in spontaneously hypertensive rats. *Hypertens Res*. 2008;(31):1619-1630. doi:10.1291/hypres.31.1619
83. Van Goor H, Van Den Born JC, Hillebrands JL, Joles JA. Hydrogen sulfide in hypertension. *Curr Opin Nephrol Hypertens*. 2016;(25):107-113. doi:10.1097/MNH.0000000000000206
84. Nagpure B V., Bian J-S. Interaction of Hydrogen Sulfide with Nitric Oxide in the Cardiovascular System. *Oxid Med Cell Longev*. 2016;2016:1-16. doi:10.1155/2016/6904327
85. Hosoki R, Matsuki N, Kimura H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun*. 1997. doi:10.1006/bbrc.1997.6878
86. Andrews KL, Lumsden NG, Farry J, Jefferis A-M, Kemp-Harper BK, Chin-Dusting JPF. Nitroxyl: a vasodilator of human vessels that is not susceptible to tolerance. *Clin Sci*. 2015. doi:10.1042/CS20140759
87. Cortese-Krott MM, Koning A, Kuhnle GGC, et al. The Reactive Species Interactome: Evolutionary Emergence, Biological Significance, and Opportunities for Redox Metabolomics and Personalized Medicine. *Antioxid Redox Signal*. 2017:684-712. doi:10.1089/ars.2017.7083
88. Cai WJ, Wang MJ, Moore PK, Jin HM, Yao T, Zhu YC. The novel proangiogenic effect of hydrogen sulfide is dependent on Akt phosphorylation. *Cardiovasc Res*. 2007;(76):29-40. doi:10.1016/j.cardiores.2007.05.026
89. Papapetropoulos A, Pyriochou A, Altaany Z, et al. Hydrogen sulfide is an endogenous stimulator of angiogenesis. *Proc Natl Acad Sci*. 2009;(106):21972-21977. doi:10.1073/pnas.0908047106
90. Szabó C, Papapetropoulos A. Hydrogen sulphide and angiogenesis: Mechanisms and applications. *Br J Pharmacol*. 2011. doi:10.1111/j.1476-5381.2010.01191.x
91. Holwerda KM, Burke SD, Faas MM, et al. Hydrogen Sulfide Attenuates sFlt1-Induced Hypertension and Renal Damage by Upregulating Vascular Endothelial Growth Factor. *J Am Soc Nephrol*. 2014;25(4):717-725. doi:10.1681/ASN.2013030291
92. Folkman J. Angiogenesis: An organizing principle for drug discovery? *Nat Rev Drug Discov*. 2007. doi:10.1038/nrd2115
93. Coletta C, Papapetropoulos A, Erdelyi K, et al. Hydrogen sulfide and nitric oxide are mutually dependent in the regulation of angiogenesis and endothelium-dependent vasorelaxation. *Proc Natl Acad Sci*. 2012. doi:10.1073/pnas.1202916109
94. Wang M-J, Cai W-J, Zhu Y-C. Mechanisms of angiogenesis: Role of hydrogen sulphide. *Clin Exp Pharmacol Physiol*. 2010. doi:10.1111/j.1440-1681.2010.05371.x
95. Geng B, Cui Y, Zhao J, et al. Hydrogen sulfide downregulates the aortic L-arginine/nitric oxide pathway in rats. *Am J Physiol Regul Integr Comp Physiol*. 2007. doi:10.1152/ajpregu.00207.2006

96. Ono K, Akaike T, Sawa T, et al. Redox chemistry and chemical biology of H₂S, hydropersulfides, and derived species: implications of their possible biological activity and utility. *Free Radic Biol Med*. 2014. doi:10.1016/j.freeradbiomed.2014.09.007
97. Predmore BL, Lefer DJ, Gojon G. Hydrogen Sulfide in Biochemistry and Medicine. *Antioxid Redox Signal*. 2012. doi:10.1089/ars.2012.4612
98. Xie Z-Z, Liu Y, Bian J-S. Hydrogen Sulfide and Cellular Redox Homeostasis. *Oxid Med Cell Longev*. 2016. doi:10.1155/2016/6043038
99. Chutkow WA, Lee RT. Thioredoxin regulates adipogenesis through thioredoxin-interacting protein (Txnip) protein stability. *J Biol Chem*. 2011. doi:10.1074/jbc.M111.267666
100. Moreno M-L, Escobar J, Izquierdo-Álvarez A, et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic Biol Med*. 2014. doi:10.1016/j.freeradbiomed.2014.01.009
101. Luo Y, He F, Hu L, et al. Transcription factor Ets1 regulates expression of thioredoxin-interacting protein and inhibits insulin secretion in pancreatic β -cells. *PLoS One*. 2014. doi:10.1371/journal.pone.0099049
102. Gan L, Johnson JA. Oxidative damage and the Nrf2-ARE pathway in neurodegenerative diseases. *Biochim Biophys Acta - Mol Basis Dis*. 2014;1842(8):1208-1218. doi:10.1016/j.bbdis.2013.12.011
103. Harvey CJ, Thimmulappa RK, Singh A, et al. Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. *Free Radic Biol Med*. 2009. doi:10.1016/j.freeradbiomed.2008.10.040
104. Sgonc R, Gruschwitz MS, Dietrich H, Recheis H, Gershwin ME, Wick G. Endothelial cell apoptosis is a primary pathogenetic event underlying skin lesions in avian and human scleroderma. *J Clin Invest*. 1996;98(3):785-792. doi:10.1172/JCI118851
105. Herrick AL. Pathogenesis of Raynaud's phenomenon. *Revmatologija*. 2005;13(3):62-65. doi:10.1093/rheumatology/keh552
106. McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am J Pathol*. 1969;(56):111-128.
107. Caramaschi P, Volpe A, Canestrini S, et al. Correlation between homocysteine plasma levels and nailfold videocapillaroscopic patterns in systemic sclerosis. *Clin Rheumatol*. 2007;26(6):902-907. doi:10.1007/s10067-006-0425-9
108. Caramaschi P, Martinelli N, Biasi D, et al. Homocysteine plasma concentration is related to severity of lung impairment in scleroderma. *J Rheumatol*. 2003.
109. Marasini B, Casari S, Bestetti A, et al. Homocysteine concentration in primary and systemic sclerosis associated Raynaud's phenomenon. *J Rheumatol*. 2000.
110. Hankey GJ, Eikelboom JW. Homocysteine and vascular disease. *Lancet*. 1999;354(176):407-413. doi:10.1016/S0140-6736(98)11058-9
111. Szamosi S, Csiki Z, Szomják E, et al. Plasma homocysteine levels, the prevalence of methylenetetrahydrofolate reductase gene C677T polymorphism and macrovascular disorders in systemic sclerosis: Risk factors for accelerated macrovascular damage? *Clin Rev Allergy Immunol*. 2009;36(2-3):145-149. doi:10.1007/s12016-008-8105-y
112. Mahalle N, Kulkarni M V., Garg MK, Naik SS. Vitamin B12 deficiency and hyperhomocysteinemia as correlates of cardiovascular risk factors in Indian subjects with coronary artery disease. *J Cardiol*. 2013;61(4):289-294. doi:10.1016/j.jjcc.2012.11.009
113. Watanabe, M.; Osada, J.; Aratani, Y.; Kluckman, K.; Reddick, R.; Malinow, M. R.; Maeda N. Mice deficient in cystathionine beta-synthase: animal models for mild and severe homocyst(e)inemia. *Proc Natl Acad Sci U S A*. 1995;92(5):1585-1589. doi:10.1073/pnas.92.5.1585

114. Eberhardt RT, Forgione MA, Cap A, et al. Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia. *J Clin Invest.* 2000;106(4):483-491. doi:10.1172/JCI8342
115. Jiang X, Yang F, Tan H, et al. Hyperhomocystinemia impairs endothelial function and eNOS activity via PKC activation. *Arterioscler Thromb Vasc Biol.* 2005;25(12):2515-2521. doi:10.1161/01.ATV.0000189559.87328.e4
116. Zhang F, Slungaard A, Vercellotti GM, Iadecola C. Superoxide-dependent cerebrovascular effects of homocysteine. *Am J Physiol.* 1998.
117. Upchurch GR, Welche GN, Fabian AJ, et al. Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. *J Biol Chem.* 1997. doi:10.1074/jbc.272.27.17012
118. Weiss N, Zhang YY, Heydrick S, Bierl C, Loscalzo J. Overexpression of cellular glutathione peroxidase rescues homocyst(e)ine-induced endothelial dysfunction. *Proc Natl Acad Sci U S A.* 2001. doi:10.1073/pnas.231428998
119. Sawle P, Foresti R, Green CJ, Motterlini R. Homocysteine attenuates endothelial haem oxygenase-1 induction by nitric oxide (NO) and hypoxia. *FEBS Lett.* 2001. doi:10.1016/S0014-5793(01)03117-9
120. Loscalzo J. The oxidant stress of hyperhomocyst(e)inemia. *J Clin Invest.* 1996;(98):5-7. doi:10.1172/JCI118776
121. Yao K. Effects of several unusual sulfur-containing amino acids on rat liver cystathionine-gamma-lyase. *Physiol Chem Phys.* 1975;7:401-408.
122. Chang L, Geng B, Yu F, et al. Hydrogen sulfide inhibits myocardial injury induced by homocysteine in rats. *Amino Acids.* 2008;(34):573-585. doi:10.1007/s00726-007-0011-8
123. Cutolo M, Pizzorni C, Tuccio M, et al. Nailfold videocapillaroscopic patterns and serum autoantibodies in systemic sclerosis. *Rheumatology.* 2004;43(6):719-726. doi:10.1093/rheumatology/keh156
124. Motegi S, Toki S, Yamada K, Uchiyama A, Ishikawa O. Elevated plasma homocysteine level is possibly associated with skin sclerosis in a series of Japanese patients with systemic sclerosis. *J Dermatol.* 2014. doi:10.1111/1346-8138.12642
125. Chiku T, Padovani D, Zhu W, Singh S, Vitvitsky V, Banerjee R. H₂S biogenesis by human cystathionine γ -lyase leads to the novel sulfur metabolites lanthionine and homolanthionine and is responsive to the grade of hyperhomocysteinemia. *J Biol Chem.* 2009;284(17):11601-11612. doi:10.1074/jbc.M808026200
126. Beyer C, Schett G, Gay S, Distler O, Distler JH. Hypoxia. Hypoxia in the pathogenesis of systemic sclerosis. *Arthritis Res Ther.* 2009;11(2):220. doi:10.1186/ar2598
127. Wang M, Guo Z, Wang S. Regulation of cystathionine γ -lyase in mammalian cells by hypoxia. *Biochem Genet.* 2014;(52):29-37. doi:10.1007/s10528-013-9624-7
128. Cindrova-Davies T, Herrera EA, Niu Y, Kingdom J, Giussani DA, Burton GJ. Reduced cystathionine γ -lyase and increased miR-21 expression are associated with increased vascular resistance in growth-restricted pregnancies: Hydrogen sulfide as a placental vasodilator. *Am J Pathol.* 2013. doi:10.1016/j.ajpath.2013.01.001
129. Wu B, Teng H, Yang G, Wu L, Wang R. Hydrogen sulfide inhibits the translational expression of hypoxia-inducible factor-1 α . *Br J Pharmacol.* 2012;167(7):1492-1505. doi:10.1111/j.1476-5381.2012.02113.x
130. Manetti M, Guiducci S, Ibba-Manneschi L, Matucci-Cerinic M. Impaired Angiogenesis in Systemic Sclerosis: The Emerging Role of the Antiangiogenic VEGF165b Splice Variant. *Trends Cardiovasc Med.* 2011;21(7):204-2010. doi:10.1016/j.tcm.2012.05.011
131. D'Alessio S, Fibbi G, Cinelli M, et al. Matrix metalloproteinase 12-dependent cleavage of urokinase receptor in systemic sclerosis microvascular endothelial cells results in impaired angiogenesis. *Arthritis Rheum.* 2004;50:3275-3285. doi:10.1002/art.20562
132. Bhatia M. Role of Hydrogen Sulfide in the Pathology of Inflammation. *Scientifica (Cairo).* 2012;2012. doi:10.6064/2012/159680

133. Li L, Bhatia M, Zhu YZ, et al. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *FASEB J*. 2005;(19):1196-1198. doi:10.1096/fj.04-3583fje
134. Zanardo RCO, Brancalione V, Distrutti E, Fiorucci S, Cirino G, Wallace JL. Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. *FASEB J*. 2006;(20):2118-2120. doi:10.1096/fj.06-6270fje
135. Zhang H, Moomchala SM, Bhatia M. Endogenous Hydrogen Sulfide Regulates Inflammatory Response by Activating the ERK Pathway in Polymicrobial Sepsis. *J Immunol*. 2008;(181):4320-4331. doi:10.4049/jimmunol.181.6.4320
136. Rinaldi L, Gobbi G, Pambianco M, Micheloni C, Mirandola P, Vitale M. Hydrogen sulfide prevents apoptosis of human PMN via inhibition of p38 and caspase 3. *Lab Invest*. 2006;(86):391-397. doi:10.1038/labinvest.3700391
137. Whiteman M, Armstrong JS, Chu SH, et al. The novel neuromodulator hydrogen sulfide: An endogenous peroxynitrite "scavenger"? *J Neurochem*. 2004;(90):765-768. doi:10.1111/j.1471-4159.2004.02617.x
138. Whiteman M, Cheung NS, Zhu YZ, et al. Hydrogen sulphide: A novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? *Biochem Biophys Res Commun*. 2005;(326):794-798. doi:10.1016/j.bbrc.2004.11.110
139. Majors A, Ehrhart LA, Pezacka EH. Homocysteine as a Risk Factor for Vascular Disease : Enhanced Collagen Production and Accumulation by Smooth Muscle Cells. *Arterioscler Thromb Vasc Biol*. 1997. doi:10.1161/01.ATV.17.10.2074
140. Mayer O, Filipovský J, Dolejšová M, Cífková R, Šimon J, Bolek L. Mild hyperhomocysteinaemia is associated with increased aortic stiffness in general population. *J Hum Hypertens*. 2006;(20):267-271. doi:10.1038/sj.jhh.1001983
141. Song K, Li Q, Yin XY, Lu Y, Liu CF, Hu LF. Hydrogen sulfide: A therapeutic candidate for fibrotic disease? *Oxid Med Cell Longev*. 2015;2015. doi:10.1155/2015/458720
142. Wang Z, Yin X, Gao L, et al. The protective effect of hydrogen sulfide on systemic sclerosis associated skin and lung fibrosis in mice model. *Springerplus*. 2016;5(1). doi:10.1186/s40064-016-2774-4
143. El-Seweid MM, Sadiq NAH, Shaker OG. Role of sulfurous mineral water and sodium hydrosulfide as potent inhibitors of fibrosis in the heart of diabetic rats. *Arch Biochem Biophys*. 2011. doi:10.1016/j.abb.2010.10.014
144. Tan G, Pan S, Li J, et al. Hydrogen sulfide attenuates carbon tetrachloride-induced hepatotoxicity, liver cirrhosis and portal hypertension in rats. *PLoS One*. 2011. doi:10.1371/journal.pone.0025943
145. Song K, Wang F, Li Q, et al. Hydrogen sulfide inhibits the renal fibrosis of obstructive nephropathy. *Kidney Int*. 2014;85(6):1318-1329. doi:10.1038/ki.2013.449
146. Abdulle AE, de Koning ME, van der Horn HJ, et al. Early Predictors for Long-Term Functional Outcome After Mild Traumatic Brain Injury in Frail Elderly Patients. *J Head Trauma Rehabil*. 2018;1. doi:10.1097/HTR.0000000000000368
147. Jung KJ, Jang HS, Kim JI, Han SJ, Park JW, Park KM. Involvement of hydrogen sulfide and homocysteine transsulfuration pathway in the progression of kidney fibrosis after ureteral obstruction. *Biochim Biophys Acta - Mol Basis Dis*. 2013;1832(12):1989-1997. doi:10.1016/j.bbadis.2013.06.015
148. Bos EM, Van Goor H, Joles JA, Whiteman M, Leuvenink HGD. Hydrogen sulfide: Physiological properties and therapeutic potential in ischaemia. *Br J Pharmacol*. 2015;172(6):1479-1493. doi:10.1111/bph.12869
149. Ryter SW, Alam J, Choi A. Heme Oxygenase-1/Carbon Monoxide: From Basic Science to Therapeutic Applications. *Physiol Rev*. 2006;(86):583-650. doi:10.1152/physrev.00011.2005
150. Szabo C, Ransy C, Módos K, et al. Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. *Br J Pharmacol*. 2014;171(8):2099-2122. doi:10.1111/bph.12369

151. Davies MJ. The oxidative environment and protein damage. *Biochim Biophys Acta - Proteins Proteomics*. 2005;1703(2):93-109. doi:10.1016/j.bbapap.2004.08.007
152. Xiao L, Dong JH, Jin S, et al. Hydrogen Sulfide Improves Endothelial Dysfunction via Downregulating BMP4/COX-2 Pathway in Rats with Hypertension. *Oxid Med Cell Longev*. 2016;2016:1-10. doi:10.1155/2016/8128957
153. Jin S, Teng X, Xiao L, et al. Hydrogen sulfide ameliorated L-NAME-induced hypertensive heart disease by the Akt/eNOS/NO pathway. *Exp Biol Med*. 2017;242(18):1831-1841. doi:10.1177/1535370217732325
154. Aghagolzadeh P, Radpour R, Bachtler M, et al. Hydrogen sulfide attenuates calcification of vascular smooth muscle cells via KEAP1/NRF2/NQO1 activation. *Atherosclerosis*. 2017;265:78-86. doi:10.1016/j.atherosclerosis.2017.08.012
155. Nicholson CK, Calvert JW. Hydrogen sulfide and ischemia-reperfusion injury. *Pharmacol Res*. 2010;62(4):289-297. doi:10.1016/j.phrs.2010.06.002
156. Zhang Q, Fu H, Zhang H, et al. Hydrogen Sulfide Preconditioning Protects Rat Liver against Ischemia/Reperfusion Injury by Activating Akt-GSK-3 β Signaling and Inhibiting Mitochondrial Permeability Transition. *PLoS One*. 2013;8(9):1-12. doi:10.1371/journal.pone.0074422
157. Johansen D, Ytrehus K, Baxter GF. Exogenous hydrogen sulfide (H₂S) protects against regional myocardial ischemia-reperfusion injury. Evidence for a role of KATP channels. *Basic Res Cardiol*. 2006;101:53-60. doi:10.1007/s00395-005-0569-9
158. Calvert JW, Jha S, Gundewar S, et al. Hydrogen sulfide mediates cardioprotection through nrf2 signaling. *Circ Res*. 2009;105(4):365-374. doi:10.1161/CIRCRESAHA.109.199919
159. Yao L-L, Huang X-W, Wang Y-G, Cao Y-X, Zhang C-C, Zhu Y-C. Hydrogen sulfide protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis by preventing GSK-3 β -dependent opening of mPTP. *Am J Physiol Heart Circ Physiol*. 2010;(298):1310-1319. doi:10.1152/ajpheart.00339.2009
160. Tripatara P, Patel NSA, Collino M, et al. Generation of endogenous hydrogen sulfide by cystathionine γ -lyase limits renal ischemia/reperfusion injury and dysfunction. *Lab Invest*. 2008;88:1038-1048. doi:10.1038/labinvest.2008.73
161. Xu Z, Prathapasinghe G, Wu N, Hwang S-Y, Siow YL, O K. Ischemia-reperfusion reduces cystathionine- β -synthase-mediated hydrogen sulfide generation in the kidney. *Am J Physiol Physiol*. 2009. doi:10.1152/ajprenal.00096.2009
162. Blackstone E, Morrison M, Roth MB. H₂S induces a suspended animation-like state in mice. *Science (80-)*. 2005;308(5721):518. doi:10.1126/science.1108581
163. Blackstone E, Roth MB. Suspended animation-like state protects mice from lethal hypoxia. *Shock*. 2007. doi:10.1097/SHK.0b013e31802e27a0
164. Whiteman M, Gooding KM, Whatmore JL, et al. Adiposity is a major determinant of plasma levels of the novel vasodilator hydrogen sulphide. *Diabetologia*. 2010;53:1722-1726. doi:10.1007/s00125-010-1761-5
165. Sun L, Jin H, Sun L, et al. Hydrogen sulfide alleviates myocardial collagen remodeling in association with inhibition of TGF- β /Smad signaling pathway in spontaneously hypertensive rats. *Mol Med*. 2015;20:503-515. doi:10.2119/molmed.2013.00096
166. Sikora M, Drapala A, Ufnal M. Exogenous hydrogen sulfide causes different hemodynamic effects in normotensive and hypertensive rats via neurogenic mechanisms. *Pharmacol Reports*. 2014;66:751-758. doi:10.1016/j.pharep.2014.04.004
167. Huang P, Chen S, Wang Y, et al. Down-regulated CBS/H₂S pathway is involved in high-salt-induced hypertension in Dahl rats. *Nitric Oxide - Biol Chem*. 2015;46:192-203. doi:10.1016/j.niox.2015.01.004
168. Tomasova L, Konopelski P, Ufnal M. Gut bacteria and hydrogen sulfide: The new old players in circulatory system homeostasis. *Molecules*. 2016;21(11):1558. doi:10.3390/molecules21111558

169. Tomasova L, Dobrowolski L, Jurkowska H, et al. Intracolonic hydrogen sulfide lowers blood pressure in rats. *Nitric Oxide - Biol Chem*. 2016;60:50-58. doi:10.1016/j.niox.2016.09.007
170. Freyer DR, Chen L, Krailo MD, et al. Effects of sodium thiosulfate versus observation on development of cisplatin-induced hearing loss in children with cancer (ACCL0431): a multicentre, randomised, controlled, open-label, phase 3 trial. *Lancet Oncol*. 2017;18(1):63-74. doi:10.1016/S1470-2045(16)30625-8
171. Whiteman M, Le Trionnaire S, Chopra M, Fox B, Whatmore J. Emerging role of hydrogen sulfide in health and disease: critical appraisal of biomarkers and pharmacological tools. *Clin Sci (Lond)*. 2011. doi:10.1042/CS20110267
172. Cicone JS, Petronis JB, Embert CD, Spector DA. Successful treatment of calciphylaxis with intravenous sodium thiosulfate. *Am J Kidney Dis*. 2004;43(6):1104-1108. doi:10.1053/j.ajkd.2004.03.018
173. Bourgeois P, De Haes P. Sodium thiosulfate as a treatment for calciphylaxis: A case series. *J Dermatolog Treat*. 2016;27(6):520-524. doi:10.3109/09546634.2016.1163316
174. Zitt E, König M, Vychytil A, et al. Use of sodium thiosulphate in a multi-interventional setting for the treatment of calciphylaxis in dialysis patients. *Nephrol Dial Transpl*. 2013;28:1232-1240. doi:10.1093/ndt/gfs548

CHAPTER

General summary, discussion,
and future perspective

GENERAL SUMMARY

Raynaud's phenomenon (RP), often the first presenting sign of SSc-related vasculopathy, is a frequently occurring disorder, and most studies in the general population reported a prevalence rate of 3%–5%.¹ Generally, vasculopathy is known to significantly impair patients' quality of life. Although vascular injury may directly arise from different etiologies, addressing risk factors associated with vasculopathy development is essential because this enables prevention. Vasculopathy plays an important role in the pathophysiology of SSc, and it is believed to be caused by abnormal neovascularization and remodeling of the vessels. Although SSc-related vasculopathy is undoubtedly complex and only partially understood,² oxidative stress has been implicated to play an important role in its initiation and progression.^{3,4} For instance, recurrent episodes of vasospasms followed by restoration of reperfusion (i.e., RP) are known to increase the production of reactive oxygen species (ROS), subsequently causing oxidative stress.⁴ Overproduction of reactive oxygen species (ROS) or a reduction in the availability of antioxidants is known to cause oxidative stress.⁵ However, essential physiological functions in cells, such as gene regulation, intermediary metabolism, and mitochondrial function, require a certain level of ROS.^{6,7} Overproduction of ROS, which overwhelms the antioxidant defense system,⁸ is known to lead to cellular damage and tissue destruction. Enhanced oxidative stress is also prominently involved in the pathogenesis of several cardiometabolic disorders, including diabetes mellitus and atherosclerotic cardiovascular disease (CVD). Obesity is an acknowledged important CVD risk factor and is also believed to promote an inflammatory and oxidative milieu.^{9–11} In contrast to the known detrimental effect of obesity on health, being underweight has been associated with RP. Moreover, previous studies in patients suffering from anorexia nervosa showed increased levels of oxidative stress in these underweight patients. These studies indicate that greater deviations from normal weight are associated with certain health risks. Part 1 of this thesis focuses on the role of low body weight, as a proxy of low fat mass, in the occurrence of RP and nailfold capillary abnormalities. Part 2 of this thesis provides an overview of the role of the redox status as an indicator of health and disease (i.e., RP).

OVERVIEW OF THE MAIN FINDINGS OF PART 1

Studies focusing on the relationship between body weight and RP are limited. Therefore, this remains poorly understood. **Chapter 2** describes our investigation of the prevalence of RP and its relationship with low body weight, involuntary weight loss, and nutritionally restricted diets. The study was conducted on 93,935 participants who participated in the Lifelines Cohort Study and completed the connective tissue disease screening questionnaire. We demonstrated that low body weight and prior involuntary weight loss are independently associated with an increased risk of RP in men and women. Additionally, a low-fat diet was found to be associated with RP in women but not in men. These associations were independent of other known etiological factors associated with RP, as well as other factors that might influence endothelial function (e.g., creatinine level, daily caloric intake, age, known CTD, smoking, and use of medication). As a result, we came to the conclusion that BMI and weight fluctuations, in patients presenting with RP, should be properly examined and monitored, as these are often overlooked causes of RP.

Given that patients with RP and anorexia nervosa were shown to have structural vascular changes via nailfold capillary microscopy (NCM),¹² we compared the nailfold capillaries of subjects with primary RP across several BMI categories (**Chapter 3**). Additionally, we investigated whether serum adiponectin, a protein hormone involved in regulating glucose levels and fatty acid breakdown and a marker of insulin resistance, was associated with NCM abnormalities. We found that underweight RP patients had NCM abnormalities more frequently (i.e., more frequently had dilated capillaries and capillary loss) than subjects with a BMI > 18.5. Additionally, patients with a higher BMI (BMI ≥ 25 kg/m²) showed a significantly shorter mean recovery time (defined as the time taken for the restoration of perfusion), as assessed with a standardized cooling experiment, than subjects with a BMI of 18–24.99 kg/m². Furthermore, our study could not demonstrate an association between adiponectin levels and the occurrence of RP. Our findings indicate that microvascular abnormalities in underweight RP patients can occur independently of the presence of a connective tissue disease and must be attributed to other mechanisms.

OVERVIEW OF THE MAIN FINDINGS OF PART 2

The reactive species interactome (which includes ROS, nitric oxide, and hydrogen sulfide) has been implicated to play an important role in RP and SSc. **Chapter 4** reviews the current literature on the role of oxidative stress in the development of SSc-related vasculopathy. This chapter discusses the role of ROS in vascular physiology and their role in SSc-related vasculopathy. We present the background of oxidative stress-related processes and discuss several studies that have highlighted the potential role of oxidative stress in the pathogenesis of SSc vasculopathy. For instance, ROS have been found to correlate with SSc vasculopathy, the formation of fibrosis, and the production of autoantibodies. Moreover, ROS can activate endothelial cells, leading to endothelial cell injury and vascular complications. These findings support the potential mechanistic role of ROS in the initiation and progression of SSc vasculopathy. Finally, we studied the use of oxidative stress-related biomarkers as indicators of disease activity and evaluated potential antioxidative strategies in SSc.

Systemic free thiol groups (R-SH, sulfhydryl groups) are believed to protect against oxidative stress through ROS scavenging.¹³ Because free thiols are easily oxidized by ROS, the depletion of serum free thiols can be used to quantify systemic oxidative stress.^{6,14,15} As a result, greater serum free thiol concentrations are often indicative of a more favourable redox condition in vivo. **Chapter 5** describes how we aimed to establish the role of serum free thiols as a predictive biomarker in the general population by investigating their relationship with the risk of cardiovascular (CV) events and all-cause mortality. We demonstrated that protein-adjusted serum free thiol concentrations are inversely associated with the risk of CV events and all-cause mortality in the general population. After adjustment for confounders, the association between protein-adjusted serum free thiol concentrations (per doubling) and CV events, as well as the association with premature all-cause mortality, remained statistically significant. Our results reveal the potential significance of serum levels of free thiols for risk stratification in primary and secondary prevention settings to reduce the CVD burden in the general population. Given our findings in **Chapter 5**, we

deem it likely that PRP and SRP patients would have higher levels of oxidative stress due to repetitive ischemia/reperfusion-related injuries. Therefore, **Chapter 6** focuses on the effects of hypothermia-induced vasoconstriction on the responsiveness of redox-related markers in subjects with SSc (n = 10), primary RP (n = 10), and healthy controls (n = 10). Circulating levels of free thiol were lower at baseline in PRP and SSc patients compared to healthy controls. These results confirm that RP patients (i.e., primary and secondary) have chronically increased levels of oxidative stress. Additionally, we observed a rapid increase in free thiols (i.e., improved redox status) in all groups during recovery following transient hypothermia. Our findings coincided with stable concentrations of the gasotransmitter end-products nitrite, nitrate, and sulfate (related to H_2S). The latter was an unexpected finding warranting further investigation. **Chapter 7** reviews the role of the reactive species H_2S in the development of SSc-related vasculopathy and as a therapeutic option in SSc. Homocysteine levels are frequently increased in SSc patients, indicating defects in the transsulfuration pathway. Hydrogen sulfide, which is known to have a number of functions, including being a powerful antioxidant and vasodilator, could therefore play a key role in the onset and progression of vasculopathy. This chapter reviewed the role of H_2S in vascular (patho-) physiology and potential disturbances in SSc. Moreover, current data from experimental animal studies were reviewed, and we discussed potential H_2S -based interventional strategies.

DISCUSSION

RP is the most prevalent disease-specific manifestation of SSc and is associated with significant disease-related morbidity. RP leads to a decreased quality of life due to increased experience of pain, impaired hand function, decreased social involvement, and increased dependence on others.¹⁶ To date, effective treatment remains an elusive challenge, leaving these patients, in some cases, with this disabling condition. An understanding of the underlying pathology of RP could support the development of novel approaches and therapeutic interventions. However, answers to some fundamental questions must be obtained to fully understand this disease. For instance, it is necessary to further investigate why some patients have RP as the first symptom of an underlying autoimmune disease, while others have primary RP and will never develop an underlying disease. Despite the event(s) initiating vascular injury in patients with SSc remaining unknown, infectious agents, cytotoxic T-cells, oxidative stress, and autoantibodies against endothelial cells have all been implicated to play an important role.¹⁷ This thesis unravels the unknowns of RP and more specifically explores the role played by several mediators (e.g., oxidative stress and body weight as a proxy for a mediator) in the occurrence of CV damage (i.e., RP).

Several hypotheses exist regarding the pathogenesis of RP and early SSc. For instance, current literature supports the vascular hypothesis, stating that early SSc begins with vascular injury, which leads to more extensive damage. This hypothesis is supported by the fact that most SSc patients present with RP as the first symptom. Additionally, RP often precedes other symptoms by several years. Campbell and LeRoy (1975) have

summarized their vascular hypothesis as “a mononuclear inflammatory response around small arteries and in the interstitium of tissues, leading to severe sclerosis of the intima of the small arteries.”¹⁸ This results in tissue induration and vascular insufficiency, which cause the signs and symptoms of SSc. Atherosclerosis is the most prevalent form of vasculopathy and shows similar pathological events to SSc-related vasculopathy. It was demonstrated that oxidative stress plays an important role in the development of atherosclerosis. In vascular cells, for example, oxidative stress is thought to promote the conversion of LDL to more atherogenic oxidized LDL (ox-LDL).^{19,20} The latter has been established as a prognostic marker for future CV events. Oxidative stress has also been found to play a substantial role in SSc-related vasculopathy, which is discussed in more depth in the following sections of this chapter. Although SSc-related vasculopathy is common in the microvasculature, macrovascular involvement is considered rare. Subclinical, early atherosclerosis in SSc has been studied previously, and discrepant outcomes have been documented. For example, studies that used the intima-media thickness (IMT) of carotid arteries as a marker of early atherosclerosis found no differences in the IMT or intraluminal diameter of the common carotid artery between SSc patients and controls.^{21–23} However, other studies have demonstrated increased arterial wall stiffness and an impaired flow-mediated dilatation at the carotid or brachial arteries in SSc patients.^{24–26} Additionally, others have shown a significant prevalence of autopsy-proven macrovascular involvement in SSc patients, which was found to occur more than 10 years earlier than in the general population.²⁷ Given that traditional risk factors of atherosclerosis were similarly distributed between the groups in the aforementioned studies, it appears unlikely that only these risk factors contribute to any putatively increased prevalence. Therefore, more attention should be given to other risk factors. The following sections discuss the role of oxidative stress and BMI in the occurrence of SSc-related vasculopathy.

ROLE OF BODY WEIGHT IN THE PATHOPHYSIOLOGY OF RAYNAUD'S PHENOMENON

Obesity is a growing health problem and is associated with an increased risk of CVD and several other medical conditions (e.g., insulin resistance, diabetes, hypertension, and sleep apnea syndrome).^{28–31} It has been well-established that obesity is an important risk factor for atherosclerosis and coronary artery disease. Additionally, a normal BMI in the presence of excess abdominal fat (i.e., central obesity) has also been implicated in increasing the risk of cardiometabolic disease.³² Although a high body weight (i.e., obesity) is generally associated with vascular damage, substantial weight loss (or a BMI < 18.5) has also been associated with several diseases (e.g., cancer, infection, and autoimmune disease) and is often caused by disease-related malnutrition. In SSc, a lack of nutrition is usually the result of gastrointestinal involvement, which occurs in 10% of patients.³³ We postulate that substantial weight loss can increase a person's susceptibility to RP. For instance, Nunes Ferreira et al. (2014) reported a case of a 46-year-old woman who developed RP 2 months after a gastric bypass and after she had lost 45% of her excess weight.³⁴ A possible explanation could be that substantial weight loss leads to a loss of

subcutaneous and perivascular adipose tissue (i.e., decreased isolation) and subsequently increases the susceptibility to vasospastic events. In support of this, it has been shown that perivascular adipose tissue alters the balance between endothelium-dependent vasodilator and vasoconstrictor substances (such as nitric oxide and endothelin-1),³⁵ which further emphasizes the importance of body weight and, more importantly, recent weight loss, in the occurrence of RP.

Chapter 2 shows that low body weight and prior involuntary weight loss are independently associated with an increased risk of RP in both men and women. It is well-known that adipose tissue can secrete non-protein biologically active mediators, including gasotransmitters (nitric oxide, carbon monoxide, and hydrogen sulfide).^{36,37} Therefore, a loss of body weight leads to a reduction in subcutaneous fat, subsequently reducing the production of the antioxidant hydrogen sulfide. Epidemiological data from several thousand individuals show that the total level of cysteine,³⁸ a key component of the enzymatic breakdown during which H₂S is produced, is positively associated with BMI and fat mass.³⁹ Other studies reported that cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE) knockout mice were characterized by a lower fat mass and showed a 34% decrease in liver glutathione.^{40,41} Additionally, patients with CBS deficiency, although rare, have been reported to develop premature arteriosclerosis and thromboembolism, which has partly been attributed to a decrease in hydrogen sulfide production. This further emphasizes the importance of the transsulfuration pathway in the occurrence of CVD.

Hydrogen sulfide, in low concentrations, is believed to have a strong antioxidant and vasodilator capacity, which is mainly due to its ROS scavenging property, as discussed in **Chapter 7**. The protective effect of H₂S in ischemia-reperfusion injury was shown in several experimental animal studies. For instance, hydrogen sulfide was shown to protect against myocardial I/R,^{42–45} hepatic I/R,^{46–48} and renal I/R injury.^{49–51} Although research on its antioxidant mechanism is sparse, evidence suggests that H₂S promotes the production of antioxidant enzymes such as GSH peroxidase and superoxide dismutase by upregulating glutathione (GSH).⁵² An ongoing study from our center is currently investigating whether the hydrogen sulfide donor, sodium thiosulfate, can attenuate the damage occurring following myocardial infarction (MI). Given the shared mechanism of MI and RP (i.e., ischemia and reperfusion injury), this study conducted in humans could even be of significance in RP.

Body weight is also believed to affect adiponectin, an adipokine produced by adipocytes as a response to a negative energy balance.⁵³ Although high adiponectin levels are generally associated with better long-term CV outcomes in healthy individuals,^{54,55} recent studies demonstrated the so-called “adiponectin paradox”. For instance, it was reported that patients with anorexia nervosa showed a tendency towards higher adiponectin levels compared to a control group.⁵⁶ Given that adiponectin not only appears to be increased in underweight patients but is also believed to have several negative effects on the vessels (e.g., impaired endothelial function, impaired vascular remodeling),⁵⁷ it is postulated that adiponectin plays a role in the occurrence of RP and nailfold changes in underweight patients.

However, as explained in **Chapter 3**, we could not demonstrate differences in serum levels of adiponectin between BMI groups. Additionally, we found no association between adiponectin and NCM. A possible explanation might be that adiponectin is only altered after extreme weight loss and is not altered in constitutionally thin subjects. However, we must acknowledge that our study could not demonstrate an association between adiponectin levels and the occurrence of RP. Therefore, larger prospective studies are required to fully elucidate the relationship between adiponectin and RP.

It could be postulated that a person's susceptibility to RP increases due to the loss of the protective effects associated with the reduction in body fat. For instance, as described in **Chapter 3**, we conducted a standardized cooling and recovery experiment in subjects in three different BMI groups (BMI < 18.5, BMI 18.5–24.99, BMI ≥ 25). We demonstrated that subjects with a BMI ≥ 25 had a significantly shorter mean recovery time than subjects who were underweight or had a healthy BMI. This finding further emphasizes the importance of body fat in thermoregulation. Although the exact role of body fat in RP must be further elucidated, **Chapter 2** and **Chapter 3** further emphasize that body weight should be evaluated in patients presenting with RP, and this should be structurally incorporated in the follow-up of RP patients, as nutritional issues are easily overlooked in association with RP.

It was reported that flow-mediated dilation improved after long-term estrogen replacement therapy in postmenopausal women with secondary RP.⁵⁸ As estrogen levels might be increased in obese patients, due to peripheral aromatization of testosterone to estrogen,³⁴ this finding reveals the possible protective role of hormonal factors in RP. The effect of hormonal changes (e.g., the menstrual cycle, use of oral contraceptives, and pregnancy) in patients with RP and healthy women on skin perfusion indicates a role of female sex hormones in this process. However, no causal effect has been found between skin blood flow and circulating levels of estrogen or progestogen. Moreover, a case series conducted by Campochiaro et al. (2018) demonstrated that hormonal modification, as part of gender transition, may be relevant in the development of SSc.⁵⁹ The authors suggested that although genetic aspects of disease susceptibility are not altered by the operation, gender change may affect epigenetic factors that interact with hormonal aspects of gender transition. In contrast to these findings, we demonstrated (**Chapter 2**) that the association between BMI, involuntary weight loss, and RP was independent of hormonal status (including hormonal treatment other than contraceptives) in women. This might indicate that the causal pathway by which body weight affects RP is not solely mediated by hormonal factors in women and that other unknown factors might have a greater role in this pathway.⁶⁰

THE ROLE OF OXIDATIVE STRESS IN THE DEVELOPMENT OF VASCULOPATHY

The vascular endothelium is an active component of the vasculature, and endothelial dysfunction is a key component in the free-radical-mediated pathogenesis of CVD.⁶¹ Current literature states that a reduction of NO bioavailability, which leads to reduced endothelium-dependent vasorelaxation, plays a significant role in endothelial dysfunction.⁶² Consistent with this statement, RP, which is characterized by recurrent ischemia and reperfusion, leads to increased levels of oxidative stress, reducing the NO bioavailability.⁶³

Although autoimmune responses and inflammation were both found to play an important role in the pathogenesis of vasculopathy in SRP, no definite initiating factors have been identified. Recently, several studies have underlined the potential role of oxidative stress in the pathogenesis of SSc vasculopathy, proposing a new aspect in the pathogenesis of this disease. In the past decade, several *in vitro* and *in vivo* studies have shown the role of overproduction of free radicals in the pathophysiology of SSc and RP.^{4,64-67} Several biomarkers of this process have been described, as reviewed in **Chapter 4**. These biomarkers include oxidized low-density lipoprotein, malondialdehyde (MDA), isoprostane, and free thiols. Free thiol groups (i.e., -SH) can be oxidized by ROS and other reactive species and are active components of the antioxidant buffer capacity. Therefore, the extracellular level of free thiols can be interpreted as a direct reflection of the overall redox status.^{13,14}

Although studies have shown that patients with primary and secondary RP have higher levels of oxidative stress,^{64,66} it remains challenging to obtain a clear understanding of whether oxidative stress levels increase during an RP attack. If so, the subsequent question would be which stage of the RP attack (ischemia vs. reperfusion) accounts for most of the generated excessive ROS. More particularly, the methods used to induce an RP attack differ between studies, and no consensus has been reached on the best method. As described in **Chapter 6**, we performed a standardized cooling and recovery experiment and concomitantly recorded photoplethysmography (PPG) to assess the perfusion of the fingertips. We demonstrated that participants with RP (both primary and secondary) had lower levels of free thiols at baseline, indicating higher levels of chronic oxidative stress. In support of this, Herrick et al. showed reduced blood levels of antioxidants such as selenium and ascorbic acid in patients with SSc, which was not caused by dietary deficiency.⁶⁸ As described in **Chapter 6**, we also demonstrated a novel feature of free thiols in these patients: the rapid free thiol rebound as a response to rewarming/reperfusion. Free thiol concentrations not only recovered during reperfusion but even reached levels exceeding baseline concentrations. This “thiol rebound” could be explained by several mechanisms. For instance, it could be hypothesized that processes related to oxidative thiol modification, as a response to hypothermia, are rapidly reversible (e.g., rapid reduction of oxidized thiols). This rapid responsiveness is particularly interesting, as it could indicate that patients with and without RP can up-regulate the antioxidant system and that this is not obstructed by the recurrent I/R in RP patients.

In addition to the importance of oxidative stress in RP, this dissertation also demonstrates the predictive and protective value of free thiols in the general population. Previous studies showed that higher levels of serum free thiols are associated with favorable outcomes in patients with chronic heart failure⁶⁹ and are associated with a beneficial outcome and CV risk profile in renal transplant recipients.⁷⁰ **Chapter 5** reports that protein-adjusted serum free thiol concentrations were inversely associated with the risk of CV events and all-cause mortality in a large population-based cohort. Many experimental animal studies revealed that typical atherosclerosis-related risk factors promote the formation of free oxygen radicals by endothelial, vascular smooth muscle, and adventitial cells. Consequently, hypercholesterolemia, diabetes, hypertension, smoking, and aging all enhance ROS production.⁷¹ Current attempts at decreasing CVD burden in the general population have focused on encouraging healthier lifestyles and the early identification of patients at increased risk of CVD. These techniques have faced a number of obstacles, including a scarcity of more reliable non-invasive predictive biomarkers for assessing the risk of cardiovascular events or mortality. Our findings suggest the potential significance of serum levels of free thiols for risk stratification in primary and secondary prevention settings to reduce the CVD burden in the general population. Our results emphasize the significance of free thiols in a broad spectrum of vascular diseases. Given that thiols are amenable to therapeutic modulation (e.g., by exogenous administration of thiol-targeted antioxidants), our results also indicate that outcomes could be modified with thiol-based therapies in high-risk patients.

DIRECTIONS FOR FUTURE RESEARCH

The exact pathophysiology of SSc-related vasculopathy remains complex and only partially understood. The main goal remains for RP patients to be free of RP attacks, to prevent the development of critical ischemia in SRP patients, and to prevent multi-organ involvement by implementing early and effective treatment. Although much progress has been made in the last decade, treatment options remain suboptimal. Several challenges must be resolved, and these are further discussed in the following sections.

PATHOPHYSIOLOGY

The prevention of SSc-related vasculopathy remains one of the greatest challenges. Several findings support the hypothesis that SSc develops due to an initial vascular insult. For instance, vasospasms (i.e., RP) occur in most SSc patients early in the disease course. Additionally, diffuse arterial intima thickening and capillary malformations are pivotal signs of SSc-related vasculopathy. Despite the clear evidence suggesting that early disease is initiated in the vasculature, characterization of the early vascular events remains challenging because patients usually present in the later stages of the disease. Moreover, the heterogeneous nature of SSc has further obstructed investigations on disease activity and progression.

It may be just as important to identify and characterize the mediators driving the vascular remodeling that lead to the typical SSc-related vasculopathy as it is to figure out the specific processes that contribute to the initial vascular damage. For instance, neovascularization is believed to be stimulated by the mobilization of stem cells from the bone marrow (into peripheral blood) and differentiation into circulating endothelial progenitor cells. Mediators produced by endothelial cells (including ET-1, connective tissue growth factor, transforming growth factor-beta, and matrix-modulating proteins) should be carefully investigated for their direct effect on (neo)vascularization and importance in activating and regulating inflammatory mediators. Although a primary vascular etiology is broadly accepted, the astonishing effects of autologous stem-cell transplantation (ASCT) on both vasculopathy and skin fibrosis suggest that immunological and inflammatory processes are also fundamental in the maintenance and progression of SSc. This hinders identifying a single target cell for SSc.

The current thesis demonstrates that weight loss is an important risk factor for RP. Future studies are needed to further unravel the effect of weight loss on the vasculature. Previous case reports demonstrated that patients who underwent gastric surgery developed RP following the operation.³⁴ Patients who undergo bariatric surgery often lose substantial amounts of weight (i.e., due to the loss of subcutaneous, visceral, and perivascular fat) in a short time and show changes in their adiponectin levels.⁷² Therefore, these patients would be highly suitable for unraveling the exact role of weight loss in the occurrence of RP. A pre and post-surgery cooling challenge could be conducted to establish whether an increase in vasoconstriction occurs in response to hypothermia following weight loss. Moreover, pre-surgery and post-surgery oxidative stress levels should also be assessed in these patients. This could further explain whether increased levels of oxidative stress are mediated through weight loss or are independent of weight changes. As described in **Chapter 2**, we also found lower levels of creatinine in subjects with RP. Although creatinine is only a surrogate marker of muscle mass, it could be hypothesized that subjects with low muscle mass might also have a higher susceptibility to developing RP. A possible explanation could be that low muscle mass reflects poor nutritional status. This is supported by the fact that low muscle mass is included in the diagnostic criteria of malnutrition.⁷³ Therefore, studies focused on lifestyle changes (e.g., healthier diet, increased exercise, and changes in body composition) should also be considered.

Future studies should also focus on disease epidemiology. For instance, observational studies will enable better characterization of risk factors. As shown in **Chapter 2**, the Lifelines cohort is an excellent cohort to investigate the prevalence of RP and early SSc. The Lifelines cohort is a multidisciplinary population-based cohort study that examines the health and health-related behaviors of 167,729 people living in the North East region of the Netherlands. More than half of the total participants completed the connective tissue disease screening questionnaire. Additionally, blood samples (serum and plasma) obtained from all participants are stored and readily available. Therefore, an essential first step in further unraveling the underlying pathophysiology of RP and early SSc would be to assess the occurrence of SSc-specific antibodies in these participants. Additionally,

the NCM patterns of these participants should be assessed. These studies will not only yield more information on the true prevalence rates of early SSc; they could also enable the development of models and tools for clinical practice that could predict disease progression. Additionally, given the prospective design of this cohort study, with extensive follow-up, the assessment of oxidative stress levels could provide more information on whether increased levels of oxidative stress are an epiphenomenon in the development of SSc or are involved in the causal pathway leading to SSc. Furthermore, studies using this large cohort could investigate whether free thiols could be used as a marker of disease progression. However, observational studies alone will not answer important questions regarding causality.

IMAGING AND VASCULAR FUNCTION MEASUREMENTS

A reliable method should be established to induce (in a controlled setting) and measure RP attacks to fully elucidate the exact pathophysiology of RP. For instance, with our approach (**Chapter 6**), we could only investigate the presence or absence of perfusion, missing the subtle differences within these stages. Peripheral arterial tonometry (PAT) would enable measurement of arterial pulse volume changes in the fingertip resulting from vasoconstriction or vasodilation. Due to the compact nature of this device, patients could even use it during their daily activities. By comparing these results with the results of the cooling experiment conducted with PAT, we could obtain valuable information on whether our aforementioned results demonstrated the effect of an RP attack or merely hypothermia. In addition to the PAT, other promising imaging modalities that could be used in the foreseeable future include optical coherence tomography, multispectral imaging, pulse wave velocity, laser Doppler speckle, photoacoustic imaging, and power Doppler ultrasound. These more accurate imaging modalities could be used to assess the effect of novel therapeutic strategies.

Furthermore, current RP studies primarily focus on the vasculature of the extremities (i.e., hands and feet), neglecting (presumably) affected vessels in different sites, such as coronary and renal vessels. Although current studies have used numerous techniques to investigate cardiac RP, large prospective studies are lacking. Future studies could conduct a cardiac MRI and a Doppler ultrasound of the renal arteries following a cooling test, as demonstrated in **Chapter 6**. This study could provide valuable information on whether vasoconstriction (following a cooling test) concomitantly occurs in different sites.

NOVEL THERAPEUTIC STRATEGIES

Given that thiols are amenable to therapeutic modulation, for example, by exogenous administration of thiol-targeted antioxidants, studies investigating the role of free thiols in RP and early SSc could be of particular interest.⁶ Although evidence exists suggesting that supplementation with some dietary antioxidants (e.g., N-acetylcysteine) is effective in increasing extracellular thiol content by reducing disulfide bonds in proteins,⁷⁴ and is associated with a decreased risk of CV death,⁷⁵ studies on SSc have been disappointing.⁴ For instance, Correa et al. (2014) demonstrated that oral N-acetylcysteine (NAC; 1800 mg/

day), which acts as a free radical scavenger, showed no vasodilator effect on the microcirculation of the hands after 4 weeks of treatment in SSc patients.⁷⁶ Additionally, Herrick et al. (2000) evaluated the effects of a combination of micronutrient antioxidants (i.e., selenium, beta-carotene, vitamin C, vitamin E, and methionine) with allopurinol in patients with limited cutaneous SSc, and they found no clinical benefit from active treatment.⁷⁷ Reactive species being part of an elaborate and highly complex redox signaling network, which primarily serves a physiological function, might explain the failure in the previously conducted antioxidant-based trials. This could explain the considerable heterogeneity of responsiveness to antioxidant-based treatment in these patients. Therefore, it would be unrealistic to believe that a single supplementation of antioxidants would give all patients substantial health benefits.

In addition to the lack of effect on disease progression and disease severity, fundamental questions remain unanswered, such as, “do antioxidant-based therapies have an intracellular thiol (or antioxidant) content increasing effect in SSc/RP patients?” For instance, if the intracellular antioxidant buffer capacity is not attenuated by the thiol-based therapy, it appears unlikely that drug-induced effects would be observed. Given the fact that mitochondria-targeted antioxidants are able to offer a greater protection against oxidative damage in the mitochondria, future studies should also focus on increasing intracellular antioxidant levels by using these targeted antioxidants.^{78–80} It could be hypothesized that on reducing ROS at the heart of the source, RP attacks will occur less frequently or be less severe due to the elimination of the self-perpetuating cycle. However, it is noteworthy that ROS also serve as important signaling molecules for several physiological functions. Given that active lowering of ROS could potentially impact the function of cells, the effect of intracellular thiol increase should be carefully investigated first.

Although autologous stem cell transplantation (ASCT) remains an extremely promising therapy, it is also known to have major limitations, including a high mortality risk, narrow applicability, and uncertainty regarding long-term outcomes. Despite these limitations, studies conducted in patients following ASCT could provide more insight into the pathophysiology of SSc and could reveal a target cell for the development of novel, more applicable, therapeutic strategies. Mesenchymal stem cell (MSC) based treatment has emerged as a novel treatment option among the innovative therapeutic methods now being explored. These cells comprise a diverse population with multilineage differentiation potential, low immunogenicity, and the capacity to produce cytokines and growth factors with pro-angiogenic, anti-inflammatory, and immunomodulatory characteristics at sites of injury. The therapeutic potential of bone-marrow-derived MSCs has been extensively investigated for SSc.⁸¹ However, their clinical use is limited by the extremely invasive nature of bone marrow harvest. In addition to the bone marrow, MSCs have also been isolated from adipose tissue and several other tissues (e.g., umbilical cord and placenta).⁸² Adipose-derived stem cells (ADSCs) have been shown to have a number of advantages, including (but not limited to) decreased donor morbidity, abundance, and fast growth.⁸³ Compared to bone-marrow-derived MSCs, ADSCs have a higher proliferation rate, reduced senescence, stronger genetic stability, and faster culture, making them a more appealing and promising source for stem cell therapy.

CONCLUDING REMARKS

In 1862, Maurice Raynaud first described “de l’asphyxie locale et de la gangrène symétrique des extrémités”, which is now known as RP. Despite considerable advances, the inability to identify patients with Raynaud’s at increased risk of developing SSc remains a problem. Any therapy started later in the disease may only attenuate disease processes and symptoms without modifying the disease course and outcome. Although underlying systemic disease should be excluded in patients presenting with RP, other easily overlooked causes should be considered, such as low body weight or recent weight loss. This dissertation reveals these important factors as well as less traditional factors such as the role of oxidative stress. Additionally, the current thesis has provided valuable insight into the significance of free thiols in the general population. Although considerable advances have been achieved since Maurice Raynaud’s initial report, much work remains. We are confident that this dissertation has significantly contributed to the current understanding of RP and paved the way for novel directions.

REFERENCES

1. Wigley FM, Herrick AL, Flavahan NA. Raynaud's Phenomenon. In: Wigley FM, Herrick AL, Flavahan NA, eds. *Raynaud's Phenomenon: A Guide to Pathogenesis and Treatment*. 1st ed. Springer-Verlag New York; 2015:21-31. doi:10.1007/978-1-4939-1526-2
2. Sunderkötter C, Riemekasten G. Pathophysiology and clinical consequences of Raynaud's phenomenon related to systemic sclerosis. *Rheumatology*. 2006;45(Suppl 3):iii33-35. doi:10.1093/rheumatology/kel280
3. Herrick AL. The pathogenesis, diagnosis and treatment of Raynaud phenomenon. *Nat Rev Rheumatol*. 2012;8(8):469-479. doi:10.1038/nrrheum.2012.96
4. Herrick AL, Matucci Cerinic M. The emerging problem of oxidative stress and the role of antioxidants in systemic sclerosis. *Clin Exp Rheumatol*. 2001;19(1):4-8.
5. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. *World Allergy Organ J*. 2012;5(1):9-19. doi:10.1097/WOX.0b013e3182439613
6. Cortese-Krott MM, Koning A, Kuhnle GGC, et al. The Reactive Species Interactome: Evolutionary Emergence, Biological Significance, and Opportunities for Redox Metabolomics and Personalized Medicine. *Antioxid Redox Signal*. 2017:684-712. doi:10.1089/ars.2017.7083
7. Jones DP, Sies H. The Redox Code. *Antioxid Redox Signal*. 2015;23(9):734-746. doi:10.1089/ars.2015.6247
8. Matés JM, Pérez-Gómez C, Núñez de Castro I. Antioxidant enzymes and human diseases. *Clin Biochem*. 1999;32(8):595-603. doi:10.1016/S0009-9120(99)00075-2
9. Higdon J V., Frei B. Obesity and oxidative stress: A direct link to CVD? *Arterioscler Thromb Vasc Biol*. 2003;23:365-367. doi:10.1161/01.ATV.0000063608.43095.E2
10. Wonisch W, Falk A, Sundl I, Winklhofer-Roob BM, Lindschinger M. Oxidative stress increases continuously with BMI and age with unfavourable profiles in males. *Aging Male*. 2012;15(3):159-165. doi:10.3109/13685538.2012.669436
11. Marseglia L, Manti S, D'Angelo G, et al. Oxidative stress in obesity: A critical component in human diseases. *Int J Mol Sci*. 2015;16(1):378-400. doi:10.3390/ijms16010378
12. De Martinis M, Sirufo MM, Ginaldi L. Raynaud's phenomenon and nailfold capillaroscopic findings in anorexia nervosa. *Curr Med Res Opin*. 2018;34(3):547-550. doi:10.1080/03007995.2017.1417828
13. Turell L, Radi R, Alvarez B. The thiol pool in human plasma: The central contribution of albumin to redox processes. *Free Radic Biol Med*. 2013;65:244-253. doi:10.1016/j.freeradbiomed.2013.05.050
14. Banne AF, Amiri A, Pero RW. Reduced level of serum thiols in patients with a diagnosis of active disease. *J Anti Aging Med*. 2003;6(4):327-334. doi:10.1089/109454503323028920
15. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol*. 2003;552(2):335-344. doi:10.1111/j.1469-7793.2003.00335.x
16. Pauling JD, Saketkoo LA, Matucci-Cerinic M, Ingegnoli F, Khanna D. The patient experience of Raynaud's phenomenon in systemic sclerosis. *Rheumatol (United Kingdom)*. 2019;58(1):18-26. doi:10.1093/rheumatology/key026
17. Kahaleh B. Vascular Disease in Scleroderma: Mechanisms of Vascular Injury. *Rheum Dis Clin North Am*. 2008;34(1):57-71. doi:10.1016/j.rdc.2007.12.004
18. Campbell PM, LeRoy EC. Pathogenesis of systemic sclerosis: A vascular hypothesis. *Semin Arthritis Rheum*. 1975;4(4):351-368. doi:10.1016/0049-0172(75)90017-7
19. Parthasarathy S, Raghavamenon A, Garelnabi MO, Santanam N. Oxidized low-density lipoprotein. *Methods Mol Biol*. 2010;(610):403-417. doi:10.1007/978-1-60327-029-8_24

20. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci U S A*. 1984;(81):3883-3887. doi:10.1073/pnas.81.12.3883
21. Cheng KS, Tiwari A, Boutin A, et al. Differentiation of primary and secondary Raynaud's disease by carotid arterial stiffness. *Eur J Vasc Endovasc Surg*. 2003;25(4):336-341. doi:10.1053/ejvs.2002.1845
22. Szűcs G, Tímár O, Szekanez Z, et al. Endothelial dysfunction precedes atherosclerosis in systemic sclerosis - Relevance for prevention of vascular complications. *Rheumatology*. 2007;46(5):759-762. doi:10.1093/rheumatology/kel426
23. Stafford L, Englert H, Gover J, Bertouch J. Distribution of macrovascular disease in scleroderma. *Ann Rheum Dis*. 1998;57(8):476-479. doi:10.1136/ard.57.8.476
24. Park JH, Sung YK, Bae SC, Song SY, Seo HS, Jun JB. Ulnar artery vasculopathy in systemic sclerosis. *Rheumatol Int*. 2009;29(9):1081-1086. doi:10.1007/s00296-009-0906-7
25. Cheng KS, Tiwari A, Boutin A, et al. Carotid and femoral arterial wall mechanics in scleroderma. *Rheumatology*. 2003;42(11):1299-1305. doi:10.1093/rheumatology/keg371
26. Colaci M, Giuggioli D, Manfredi A, et al. Aortic pulse wave velocity measurement in systemic sclerosis patients. *Reumatismo*. 2012;64(6):360-367. doi:10.4081/reumatismo.2012.360
27. Belch JJ, McSwiggan S, Lau C. Macrovascular disease in systemic sclerosis: the tip of an iceberg? *Rheumatology (Oxford)*. 2008;47(Suppl 5):v16-17.
28. Polsky S, Ellis SL. Obesity, insulin resistance, and type 1 diabetes mellitus. *Curr Opin Endocrinol Diabetes Obes*. 2015;22(4):277-282. doi:10.1097/MED.0000000000000170
29. Apovian CM. Obesity: definition, comorbidities, causes, and burden. *Am J Manag Care*. 2016;22(7 Suppl):s176-185.
30. Meurling IJ, Shea DO, Garvey JF. Obesity and sleep: A growing concern. *Curr Opin Pulm Med*. 2019;25(6):602-608. doi:10.1097/MCP.0000000000000627
31. Seravalle G, Grassi G. Obesity and hypertension. *Pharmacol Res*. 2017;(122):1-7. doi:10.1016/j.phrs.2017.05.013
32. Sahakyan KR, Somers VK, Rodriguez-Escudero JP, et al. Normal-weight central obesity: Implications for total and cardiovascular mortality. *Ann Intern Med*. 2015;163(11):827-835. doi:10.7326/M14-2525
33. Hughes M, Heal C, Siegert E, et al. Significant weight loss in systemic sclerosis: A study from the EULAR Scleroderma Trials and Research (EUSTAR) database. *Ann Rheum Dis*. 2020;79(8):1123-1125. doi:10.1136/annrheumdis-2020-217035
34. Nunes Ferreira A, Aldersley R, Efthimiou E. A curious onset of Raynaud's phenomenon after gastric bypass: A case report. *Surg Obes Relat Dis*. 2014;10(6):e109-e111. doi:10.1016/j.soard.2014.01.011
35. Houben AJ, Eringa EC, Jonk AM, Serne EH, Smulders YM, Stehouwer CD. Perivascular Fat and the Microcirculation: Relevance to Insulin Resistance, Diabetes, and Cardiovascular Disease. *Curr Cardiovasc Risk Rep*. 2012;6(1):80-90. doi:10.1007/s12170-011-0214-0
36. Luo L, Liu M. Adipose tissue in control of metabolism. *J Endocrinol*. 2016;231(31):R77-R99. doi:10.1530/JOE-16-0211
37. Bektowski J, Jamroz-Wisniewska A. Hydrogen sulfide in the adipose tissue-physiology, pathology and a target for pharmacotherapy. *Molecules*. 2017;22(1):63. doi:10.3390/molecules22010063
38. Carter RN, Morton NM. Cysteine and hydrogen sulphide in the regulation of metabolism: Insights from genetics and pharmacology. *J Pathol*. 2016;238(2):321-332. doi:10.1002/path.4659
39. Elshorbagy AK, Gjesdal CG, Nurk E, et al. Cysteine, homocysteine and bone mineral density: A role for body composition? *Bone*. 2009;44(5):954-958. doi:10.1016/j.bone.2008.12.018
40. Gupta S, Kruger WD. Cystathionine beta-synthase deficiency causes fat loss in mice. *PLoS One*.

- 2011;6(11):e27598. doi:10.1371/journal.pone.0027598
41. Mani S, Yang G, Wang R. A critical life-supporting role for cystathionine γ -lyase in the absence of dietary cysteine supply. *Free Radic Biol Med*. 2011;50(10):1280-1287. doi:10.1016/j.freeradbiomed.2011.01.038
 42. Issa K, Kimmoun A, Collin S, et al. Compared effects of inhibition and exogenous administration of hydrogen sulphide in ischaemia-reperfusion injury. *Crit Care*. 2013;17:article R129. doi:10.1186/cc12808
 43. Sivarajah A, Collino M, Yasin M, et al. Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a rat model of regional myocardial I/R. *Shock*. 2009;31(3):267-274. doi:10.1097/SHK.0b013e318180ff89
 44. Sun WH, Liu F, Chen Y, Zhu YC. Hydrogen sulfide decreases the levels of ROS by inhibiting mitochondrial complex IV and increasing SOD activities in cardiomyocytes under ischemia/reperfusion. *Biochem Biophys Res Commun*. 2012;421(2):164-169. doi:10.1016/j.bbrc.2012.03.121
 45. Zhou Y, Wang D, Gao X, Lew K, Richards AM, Wang P. mTORC2 phosphorylation of Akt1: A possible mechanism for hydrogen sulfide-induced cardioprotection. *PLoS One*. 2014;9(6). doi:10.1371/journal.pone.0099665
 46. Chen Y, Liu Z, Xie X. Hydrogen sulphide attenuates renal and cardiac injury after total hepatic ischemia and reperfusion. *J Surg Res*. 2010;164(2):e305-e313. doi:10.1016/j.jss.2010.08.010
 47. Kang K, Zhao M, Jiang H, Tan G, Pan S, Sun X. Role of hydrogen sulfide in hepatic ischemia-reperfusion-induced injury in rats. *Liver Transplant*. 2009;15(10):1306-1314. doi:10.1002/lt.21810
 48. Jha S, Calvert JW, Duranski MR, Ramachandran A, Lefer DJ. Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: Role of antioxidant and antiapoptotic signaling. *Am J Physiol - Hear Circ Physiol*. 2008;295(2):H801-H806. doi:10.1152/ajpheart.00377.2008
 49. Bos EM, Wang R, Snijder PM, et al. Cystathionine γ -lyase protects against renal ischemia/reperfusion by modulating oxidative stress. *J Am Soc Nephrol*. 2013;24(5):759-770. doi:10.1681/ASN.2012030268
 50. Hunter JP, Hosgood SA, Patel M, Rose R, Read K, Nicholson ML. Effects of hydrogen sulphide in an experimental model of renal ischaemia-reperfusion injury. *Br J Surg*. 2012;99(12):1665-1671. doi:10.1002/bjs.8956
 51. Lobb I, Zhu J, Liu W, Haig A, Lan Z, Sener A. Hydrogen sulfide treatment ameliorates long-term renal dysfunction resulting from prolonged warm renal ischemia-reperfusion injury. *J Can Urol Assoc*. 2014;8(5-6):E413-E418. doi:10.5489/cuaj.1694
 52. Jain SK, Huning L, Micinski D. Hydrogen sulfide upregulates glutamate-cysteine ligase catalytic subunit, glutamate-cysteine ligase modifier subunit, and glutathione and inhibits interleukin-1 β secretion in monocytes exposed to high glucose levels. *Metab Syndr Relat Disord*. 2014;12(5):299-302. doi:10.1089/met.2014.0022
 53. Baker JF, Newman AB, Kanaya A, et al. The Adiponectin Paradox in the Elderly: Associations with Body Composition, Physical Functioning, and Mortality. *Journals Gerontol - Ser A Biol Sci Med Sci*. 2019;74(2):247-253. doi:10.1093/gerona/gly017
 54. Pischon T, Hu FB, Girman CJ, et al. Plasma total and high molecular weight adiponectin levels and risk of coronary heart disease in women. *Atherosclerosis*. 2011;219:322-329. doi:10.1016/j.atherosclerosis.2011.07.011
 55. Pischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB, Rimm EB. Plasma Adiponectin Levels and Risk of Myocardial Infarction in Men. *J Am Med Assoc*. 2004;291(1730-1737). doi:10.1001/jama.291.14.1730
 56. Pannacciulli N, Vettor R, Milan G, et al. Anorexia nervosa is characterized by increased adiponectin plasma levels and reduced nonoxidative glucose metabolism. *J Clin Endocrinol Metab*. 2003;88:1748-1752. doi:10.1210/jc.2002-021215
 57. Hopkins TA, Ouchi N, Shibata R, Walsh K. Adiponectin actions in the cardiovascular system. *Cardiovasc Res*. 2007;74(1):11-18. doi:10.1016/j.cardiores.2006.10.009

58. Lekakis J, Papamichael C, Mavrikakis M, Voutsas A, Stamatelopoulos S. Effect of long-term estrogen therapy on brachial arterial endothelium-dependent vasodilation in women with Raynaud's phenomenon secondary to systemic sclerosis. *Am J Cardiol*. 1998;82(0002-9149 LA-eng PT-Clinical Trial PT-Journal Article PT-Randomized Controlled Trial):1555-1557, A8.
59. Campochiaro C, Host L V., Ong VH, Denton CP. Development of systemic sclerosis in transgender females: A case series and review of the literature. *Clin Exp Rheumatol*. 2018;(36 (suppl 113)):S50-S52.
60. Kamitaki N, Sekar A, Handsaker RE, et al. Complement genes contribute sex-biased vulnerability in diverse disorders. *Nature*. 2020;582:577-581. doi:10.1038/s41586-020-2277-x
61. Elahi MM, Kong YX, Matata BM. Oxidative stress as a mediator of cardiovascular disease. *Oxid Med Cell Longev*. 2009;2(5):259-269. doi:10.4161/oxim.2.5.9441
62. Shah AM, Channon KM. Free radicals and redox signalling in cardiovascular disease. *Heart*. 2004;90(5):486-487. doi:10.1136/hrt.2003.029389
63. Cooke JP, Marshall JM. Mechanisms of Raynaud's disease. *Vasc Med*. 2005;(10):293-307. doi:10.1191/1358863x05vm639ra
64. Biondi R, Coaccioli S, Lattanzi S, Puxeddu A, Papini M. Oxidant/antioxidant status in patients with Raynaud's disease. *Clin Ter*. 2008;159(2):77-81.
65. Dosoki H, Stegemann A, Taha M, et al. Targeting of NADPH oxidase in vitro and in vivo suppresses fibroblast activation and experimental skin fibrosis. *Exp Dermatol*. 2017;26(1):73-81. doi:10.1111/exd.13180
66. Ogawa F, Shimizu K, Muroi E, et al. Serum levels of 8-isoprostane, a marker of oxidative stress, are elevated in patients with systemic sclerosis. *Rheumatology*. 2006;45(7):815-818. doi:10.1093/rheumatology/kei012
67. Iwata Y, Ogawa F, Komura K, et al. Autoantibody against peroxiredoxin I, an antioxidant enzyme, in patients with systemic sclerosis: Possible association with oxidative stress. *Rheumatology*. 2007;46(5):790-795. doi:10.1093/rheumatology/kem010
68. Herrick AL, Worthington H, Rieley F, et al. Dietary intake of micronutrient antioxidants in relation to blood levels in patients with systemic sclerosis. *J Rheumatol*. 1996;23:650-653.
69. Koning AM, Meijers WC, Pasch A, et al. Serum free thiols in chronic heart failure. *Pharmacol Res*. 2016;111:452-458. doi:10.1016/j.phrs.2016.06.027
70. Frenay ARS, de Borst MH, Bachtler M, et al. Serum free sulfhydryl status is associated with patient and graft survival in renal transplant recipients. *Free Radic Biol Med*. 2016;(93):345-351. doi:10.1016/j.freeradbiomed.2016.08.024
71. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol*. 2003;91:7a-11a. doi:10.1016/S0002-9149(02)03144-2
72. Swarbrick MM, Austrheim-Smith IT, Stanhope KL, et al. Circulating concentrations of high-molecular-weight adiponectin are increased following Roux-en-Y gastric bypass surgery. *Diabetologia*. 2006;49(11):2552-2558. doi:10.1007/s00125-006-0452-8
73. Deutz NEP, Ashurst I, Ballesteros MD, et al. The Underappreciated Role of Low Muscle Mass in the Management of Malnutrition. *J Am Med Dir Assoc*. 2019;20(1):22-27. doi:10.1016/j.jamda.2018.11.021
74. Atkuri KR, Mantovani JJ, Herzenberg LA, Herzenberg LA. N-Acetylcysteine-a safe antidote for cysteine/glutathione deficiency. *Curr Opin Pharmacol*. 2007;7(4):355-359. doi:10.1016/j.coph.2007.04.005
75. Loffredo L, Perri L, Di Castelnuovo A, Iacoviello L, De Gaetano G, Violi F. Supplementation with vitamin E alone is associated with reduced myocardial infarction: A meta-analysis. *Nutr Metab Cardiovasc Dis*. 2015;25(4):354-363. doi:10.1016/j.numecd.2015.01.008

76. Correa MJU, Mariz HA, Andrade LEC, Kayser C. Oral N-acetylcysteine in the treatment of Raynaud's phenomenon secondary to systemic sclerosis: A randomized, double-blind, placebo-controlled clinical trial. *Rev Bras Reumatol*. 2014;54(6):452-458. doi:10.1016/j.rbre.2014.09.001
77. Herrick AL, Hollis S, Schofield D, et al. A double-blind placebo-controlled trial of antioxidant therapy in limited cutaneous systemic sclerosis. *Clin Exp Rheumatol*. 2000;18(3):349-356.
78. Gioscia-Ryan RA, LaRocca TJ, Sindler AL, Zigler MC, Murphy MP, Seals DR. Mitochondria-targeted antioxidant (MitoQ) ameliorates age-related arterial endothelial dysfunction in mice. *J Physiol*. 2014;592(12):2549-2561. doi:10.1113/jphysiol.2013.268680
79. Oyewole AO, Wilmot MC, Fowler M, Birch-Machin MA. Comparing the effects of mitochondrial targeted and localized antioxidants with cellular antioxidants in human skin cells exposed to UVA and hydrogen peroxide. *FASEB J*. 2014;28(1):485-494. doi:10.1096/fj.13-237008
80. Oyewole AO, Birch-Machin MA. Mitochondria-targeted antioxidants. *FASEB J*. 2015;29(12):4766-4771. doi:10.1096/fj.15-275404
81. Jimenez S a. Role of endothelial to mesenchymal transition in the pathogenesis of the vascular alterations in systemic sclerosis. *ISRN Rheumatol*. 2013;2013:835948. doi:10.1155/2013/835948
82. Maria ATJ, Maumus M, Le Quellec A, Jorgensen C, Noël D, Guilpain P. Adipose-Derived Mesenchymal Stem Cells in Autoimmune Disorders: State of the Art and Perspectives for Systemic Sclerosis. *Clin Rev Allergy Immunol*. 2017;52(2):234-259. doi:10.1007/s12016-016-8552-9
83. Rosa I, Romano E, Fioretto BS, Matucci-Cerinic M, Manetti M. Adipose-derived stem cells: Pathophysiologic implications vs therapeutic potential in systemic sclerosis. *World J Stem Cells*. 2021;13(1):30-48. doi:10.4252/wjsc.v13.i1.30

Nederlandse samenvatting

Dankwoord

Curriculum vitae

Bibliography

NEDERLANDSE SAMENVATTING

Het fenomeen van Raynaud (RP) is een veelvoorkomende stoornis, waarbij de handen en/of voeten aanvalsgewijs verkleuren als reactie op koude of emotionele stress. De prevalentie in de algemene populatie wordt geschat tussen de 3% en 5%.¹ In de minderheid van de gevallen is RP het eerste symptoom van een onderliggende systeemziekte, zoals systemische sclerose (SSc). Vasculopathie speelt een belangrijke rol in de pathofysiologie van SSc en vermoed wordt dat dit veroorzaakt wordt door abnormale neovascularisatie en remodelering van de bloedvaten. Hoewel SSc-gerelateerde vasculopathie zonder enige twijfel complex is en slechts gedeeltelijk begrepen wordt,² blijkt oxidatieve stress een belangrijke rol te spelen bij het ontstaan en de progressie van SSc.^{3,4} Zo is bekend dat terugkerende episodes van vasospasmen, gevolgd door herstel van perfusie (dat wil zeggen RP) de productie van reactieve zuurstofverbindingen (ROS) doen toenemen, wat vervolgens oxidatieve stress veroorzaakt.⁴ Oxidatieve stress wordt veroorzaakt door de overproductie van ROS of een lagere beschikbaarheid van antioxidanten.⁵ Een bepaalde hoeveelheid ROS is echter noodzakelijk voor verschillende fysiologische processen in cellen, zoals de mitochondriale functie.^{6,7} Het is bekend dat overmatige productie van ROS, wat het antioxidant verdedigingssysteem overweldigt,⁸ leidt tot celschade en weefseldestructie.

Een toename van de oxidatieve stress is ook in hoge mate betrokken bij de pathogenese van verschillende cardiometabole ziekten, waaronder diabetes mellitus en hart- en vaatziekten (HVZ). Obesitas is een belangrijke risicofactor voor hart- en vaatziekten en er wordt ook aangenomen dat obesitas een inflammatoire en oxidatieve omgeving bevordert.⁹⁻¹¹ In tegenstelling tot het bekende schadelijke effect van obesitas op de gezondheid, wordt ondergewicht juist geassocieerd met het voorkomen van RP. Eerdere studies bij patiënten die lijden aan anorexia nervosa hebben aangetoond dat deze patiënten met ernstig ondergewicht vaak een verhoogd oxidatieve stress gehalte hebben. Deze studies geven aan dat grote afwijkingen van een normaal gewicht gepaard gaan met bepaalde gezondheidsrisico's. Deel 1 van deze thesis focust zich op de associatie tussen een laag lichaamsgewicht en het voorkomen van RP en afwijkingen van de nagelriemcapillairen. Deel 2 van deze thesis geeft vervolgens een overzicht van de rol van de redoxstatus, als een indicator voor gezondheid en ziekte (dat wil zeggen RP). In **hoofdstuk 1** is een algemene introductie opgenomen en zijn de doelen van dit proefschrift beschreven.

Het aantal studies dat gericht is op het verband tussen lichaamsgewicht en RP is zeer beperkt, met als gevolg dat de relatie tussen ondergewicht en RP tot op heden nog slecht wordt begrepen. **Hoofdstuk 2** beschrijft ons onderzoek naar de prevalentie van RP en het verband met een laag lichaamsgewicht en onvrijwillig gewichtsverlies. We hebben aangetoond dat een laag lichaamsgewicht en onvrijwillig gewichtsverlies onafhankelijk geassocieerd zijn met een verhoogd risico op RP bij mannen en vrouwen. Tevens is aangetoond dat een vetarm dieet geassocieerd wordt met RP bij vrouwen, maar niet bij mannen. Deze associaties waren onafhankelijk van andere factoren die geassocieerd zijn met RP en die invloed kunnen uitoefenen op de endotheliale werking (bijvoorbeeld creatininespiegel, dagelijkse calorie-inname, leeftijd, bindweefselandoening in de

voorgeschiedenis, roken en medicijngebruik). In **hoofdstuk 2** wordt geconcludeerd dat BMI en gewichtsverandering structureel geëvalueerd dienen te worden bij patiënten die zich presenteren met RP, aangezien deze oorzaken vaak over het hoofd worden gezien.

In **hoofdstuk 3** is aandacht besteed aan de rol van BMI op de aanwezigheid van afwijkingen van de nagelriemcapillairen in patiënten met RP. Tevens is onderzocht of serum adiponectine, een eiwithormoon dat betrokken is bij het reguleren van de glucosespiegels en de afbraak van vetzuren en een marker voor insulineresistentie, geassocieerd is met nagelriemcapillairmicroscopie (NCM) afwijkingen. Daarbij is vastgesteld dat RP-patiënten met ondergewicht vaker NCM-afwijkingen hadden (dat wil zeggen: frequenter gedilateerde haarvaten en capillair verlies hadden) dan personen met een BMI > 18.5. Bovendien is aangetoond dat patiënten met een hoger BMI (BMI $\geq$ 25 kg/m²) een significant kortere hersteltijd hadden (gedefinieerd als de tijd die nodig is voor het herstellen van de perfusie), zoals geëvalueerd met een gestandaardiseerd afkoelingsexperiment, dan personen met een BMI van 18–24,99 kg/m². Verder kon deze studie geen verband aantonen tussen adiponectinespiegels en het voorkomen van RP. Onze bevindingen geven aan dat microvasculaire aandoeningen bij RP-patiënten met ondergewicht zich onafhankelijk van de aanwezigheid van een bindweefselaandoening kunnen voordoen en toegeschreven dienen te worden aan andere mechanismen.

Al eerder is aangetoond dat ROS, stikstofoxide en waterstofsulfide een belangrijke rol spelen in RP en SSc. **Hoofdstuk 4** behandelt de huidige literatuur over de rol van oxidatieve stress in de ontwikkeling van SSc-gerelateerde vasculopathie. Dit hoofdstuk bespreekt de rol van ROS in de vasculaire fysiologie en in SSc-gerelateerde vasculopathie. We bespreken de achtergrond van oxidatieve stressgerelateerde processen (bijvoorbeeld letsels van endotheelcellen, auto-immuniteit, ontsteking en remodellering van de vaatwand) en verschillende studies die de mogelijke rol van oxidatieve stress bij de pathogenese van SSc vasculopathie verduidelijkt hebben. Zo is vastgesteld dat ROS selectief endotheelcellen activeren, wat leidt tot letsels van endotheelcellen en vasculaire complicaties. Deze bevindingen ondersteunen de potentieel mechanische rol van ROS bij het ontstaan en de progressie van SSc vasculopathie. Tot slot is het gebruik van oxidatieve stressgerelateerde markers als klinische biomarkers voor ziekteactiviteit bestudeerd en zijn mogelijke anti-oxidatieve strategieën bij SSc geëvalueerd.

Aangenomen wordt dat systemische vrije thiolgroepen (R-SH, sulfhydrylgroepen) bescherming bieden tegen oxidatieve stress door het 'wegvangen' van ROS.¹² Aangezien vrije thiolen eenvoudig geoxideerd worden door ROS, kan systemische oxidatieve stress eenvoudig gemeten worden als depletie van serumvrije thiolen.^{6,13,14} Hoge concentraties van serumvrije thiolen zijn gewoonlijk representatief voor een gunstigere redoxstatus *in vivo*. **Hoofdstuk 5** beschrijft de rol van serumvrije thiolen als voorspellende biomarker voor cardiovasculaire ziekten en mortaliteit in de algemene populatie. We hebben aangetoond dat serumvrije thiolenconcentraties omgekeerd geassocieerd zijn met een verhoogd risico op HVZ en mortaliteit. Na aanpassing voor confounders bleef dit verband statistisch significant. Onze resultaten duiden op het mogelijke belang van

vrije thiolen voor risicostratificatie als primaire en secundaire preventieparameters om HVZ te beperken in de algemene bevolking. Gezien onze bevindingen in **hoofdstuk 5**, achten we het waarschijnlijk dat patiënten met RP hogere concentraties van oxidatieve stress hebben vanwege repetitieve ischemie/reperfusie-gerelateerde letsels. Daarom focust **hoofdstuk 6** zich op de effecten van hypothermie-geïnduceerde vasoconstrictie op de vrije thiolenconcentratie in deelnemers met SSc (n = 10), primaire RP (n = 10) en gezonde controles (n = 10). Circulerende gehalten van vrije thiolen waren lager op baseline in PRP- en SSc-patiënten, in vergelijking met de gezonde controlegroep. Deze resultaten bevestigen dat RP-patiënten (primair en secundair) chronisch verhoogde concentraties van oxidatieve stress hebben. Ook toonden wij aan dat er sprake is van een snelle toename van vrije thiolen (d.w.z. een verbeterde redoxstatus) in alle groepen tijdens de herstelperiode (na tijdelijke hypothermie). Onze bevindingen gingen gepaard met stabiele concentraties van gasotransmitter-eindproducten nitriet, nitraat en sulfaat (gerelateerd met H_2S). De snelle stijging van vrije thiolen in de herstelperiode was een onverwachte bevinding die verder onderzoek vereist.

In **hoofdstuk 7** wordt de rol van waterstofsulfide (H_2S) besproken bij de ontwikkeling van SSc-gerelateerde vasculopathie en als therapeutische optie in SSc. In dit hoofdstuk wordt de rol van H_2S in de vasculaire (patho)fysiologie en mogelijke verstoringen in SSc-patiënten besproken. Bovendien zijn de resultaten van eerdere experimentele dierenstudies onderzocht en komen de mogelijke H_2S -gebaseerde interventionele strategieën aan bod.¹⁵⁻¹⁹ Bij patiënten met SSc wordt vaak een verhoogd gehalte van homocysteïne (dat omgezet wordt naar H_2S) gevonden, wat wijst op defecten in de transsulfuratie-route. Er zijn verschillende effecten bekend van H_2S , zoals een sterk antioxidant en vasodilerend effect. Onze hypothese is dat H_2S potentieel een belangrijke rol kan spelen in het beperken van de ziekteprogressie in SSc. De bewijzen voor deze hypothese komen in **hoofdstuk 7** aan bod.

CONCLUSIE

Ondanks aanzienlijke ontwikkelingen blijft het onvermogen om patiënten met SSc vroegtijdig te identificeren een groot probleem. Elke behandeling die later in het ziektebeloop gestart wordt, beperkt mogelijk enkel de ziekteprocessen en symptomen, zonder het ziektebeloop en uitkomsten te veranderen. Hoewel een onderliggende systemische aandoening uitgesloten dient te worden bij patiënten die zich presenteren met RP, dienen andere oorzaken (die gemakkelijk over het hoofd gezien worden) uitgesloten te worden, zoals een laag lichaamsgewicht of recent gewichtsverlies. In dit proefschrift worden deze factoren belicht, evenals traditionele factoren zoals de rol van oxidatieve stress. Ook biedt dit proefschrift waardevolle inzichten in het belang van vrije thiolen als significante voorspeller van HVZ in de algemene populatie. Hoewel er in de laatste jaren een aanzienlijke vooruitgang is geboekt met onderzoek naar de pathogenese van RP en SSc, dient nog veel werk verzet te worden. We zijn ervan overtuigd dat dit proefschrift significant bijgedragen heeft aan het huidige begrip van RP en de weg heeft vrijgemaakt om nieuwe richtingen in te slaan.

REFERENTIES

1. Wigley FM, Herrick AL, Flavahan NA. Raynaud's Phenomenon. In: Wigley FM, Herrick AL, Flavahan NA, eds. *Raynaud's Phenomenon: A Guide to Pathogenesis and Treatment*. 1st ed. Springer-Verlag New York; 2015:21-31. doi:10.1007/978-1-4939-1526-2
2. Sunderkötter C, Riemekasten G. Pathophysiology and clinical consequences of Raynaud's phenomenon related to systemic sclerosis. *Rheumatology*. 2006;45(Suppl 3):iii33-35. doi:10.1093/rheumatology/kel280
3. Herrick AL. The pathogenesis, diagnosis and treatment of Raynaud phenomenon. *Nat Rev Rheumatol*. 2012;8(8):469-479. doi:10.1038/nrrheum.2012.96
4. Herrick AL, Matucci Cerinic M. The emerging problem of oxidative stress and the role of antioxidants in systemic sclerosis. *Clin Exp Rheumatol*. 2001;19(1):4-8.
5. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. *World Allergy Organ J*. 2012;5(1):9-19. doi:10.1097/WOX.0b013e3182439613
6. Cortese-Krott MM, Koning A, Kuhnle GGC, et al. The Reactive Species Interactome: Evolutionary Emergence, Biological Significance, and Opportunities for Redox Metabolomics and Personalized Medicine. *Antioxid Redox Signal*. 2017:684-712. doi:10.1089/ars.2017.7083
7. Jones DP, Sies H. The Redox Code. *Antioxid Redox Signal*. 2015;23(9):734-746. doi:10.1089/ars.2015.6247
8. Matés JM, Pérez-Gómez C, Núñez de Castro I. Antioxidant enzymes and human diseases. *Clin Biochem*. 1999;32(8):595-603. doi:10.1016/S0009-9120(99)00075-2
9. Higdon J V., Frei B. Obesity and oxidative stress: A direct link to CVD? *Arterioscler Thromb Vasc Biol*. 2003;23:365-367. doi:10.1161/01.ATV.0000063608.43095.E2
10. Wonisch W, Falk A, Sundl I, Winklhofer-Roob BM, Lindschinger M. Oxidative stress increases continuously with BMI and age with unfavourable profiles in males. *Aging Male*. 2012;15(3):159-165. doi:10.3109/13685538.2012.669436
11. Marseglia L, Manti S, D'Angelo G, et al. Oxidative stress in obesity: A critical component in human diseases. *Int J Mol Sci*. 2015;16(1):378-400. doi:10.3390/ijms16010378
12. Turell L, Radi R, Alvarez B. The thiol pool in human plasma: The central contribution of albumin to redox processes. *Free Radic Biol Med*. 2013;65:244-253. doi:10.1016/j.freeradbiomed.2013.05.050
13. Banne AF, Amiri A, Pero RW. Reduced level of serum thiols in patients with a diagnosis of active disease. *J Anti Aging Med*. 2003;6(4):327-334. doi:10.1089/109454503323028920
14. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol*. 2003;552(2):335-344. doi:10.1111/j.1469-7793.2003.00335.x
15. Snijder PM, Frenay ARS, Koning AM, et al. Sodium thiosulfate attenuates angiotensin II-induced hypertension, proteinuria and renal damage. *Nitric Oxide - Biol Chem*. 2014;42:87-98. doi:10.1016/j.niox.2014.10.002
16. Wang M, Guo Z, Wang S. Regulation of cystathionine γ -lyase in mammalian cells by hypoxia. *Biochem Genet*. 2014;52:29-37. doi:10.1007/s10528-013-9624-7
17. Blackstone E, Morrison M, Roth MB. H2S induces a suspended animation-like state in mice. *Science (80-)*. 2005;308(5721):518. doi:10.1126/science.1108581
18. Tomasova L, Konopelski P, Ufnal M. Gut bacteria and hydrogen sulfide: The new old players in circulatory system homeostasis. *Molecules*. 2016;21(11):1558. doi:10.3390/molecules21111558
19. Bełtowski J. Hydrogen sulfide in pharmacology and medicine - An update. *Pharmacol Reports*. 2015;67(2015):647-658. doi:10.1016/j.pharep.2015.01.005

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CURRICULUM VITAE

Amaal Eman Abdulle werd op 16 februari 1992 geboren in Delfzijl. Ze behaalde haar VWO-diploma in 2010 aan het Ommelander College te Appingedam. Aansluitend startte ze met de studie geneeskunde aan de Rijksuniversiteit Groningen. Ze liep co-schappen in het Scherper ziekenhuis en het UMCG. De semi-arts stage verrichte ze bij de interne geneeskunde en de SEH in het OZG. Na het afronden van de studie begin 2017 startte ze als arts-onderzoeker bij de afdeling vasculaire geneeskunde van het UMCG. Tijdens haar promotie-traject was ze ook, onder supervisie van dr. D.J. Mulder, werkzaam als ANIOS interne geneeskunde (polikliniek OZG & UMCG).

Amaal is in mei 2020 gestart met de opleiding tot internist en in dat kader werkzaam in het Deventer Ziekenhuis.

BIBLIOGRAPHY

- A.E. Abdulle**, A.M. van Roon, A.J. Smit, A. Pasch, M. van Meurs, H. Bootsma, S.J.L. Bakker, M.Y. Said, B.O. Fernandez, M. Feelisch, H. van Goor, D.J. Mulder. Rapid free thiol rebound is a physiological response following cold-induced vasoconstriction in healthy humans, primary Raynaud and systemic sclerosis. *Physiological Reports*. 2019;7:e14017.
- A.E. Abdulle**, S. Arends, H. van Goor, E. Brouwer, A.M. van Roon, J. Westra, A.L. Herrick, K. de Leeuw, D.J. Mulder. Low body weight and involuntary weight loss are associated with Raynaud's phenomenon in both men and women. *Scandinavian Journal of Rheumatology*. 2021 Mar;50(2):153-160.
- A.E. Abdulle**, A.R. Bourgonje, L.M. Kieneker, A.M. Koning, S. la Bastide-van Gemert, M.L.C. Bulthuis, G. Dijkstra, K.N. Faber, R.P.F. Dullaart, S.J.L. Bakker, R.O.B. Gans, R.T. Gansevoort, D.J. Mulder, A. Pasch, H. van Goor. Serum free thiols predict cardiovascular events and all-cause mortality in the general population: a prospective cohort study. *BMC Medicine*. 2020 May 27;18(1):130.
- A.E. Abdulle**, H. van Goor, D.J. Mulder. Hydrogen Sulfide: A Therapeutic Option in Systemic Sclerosis. *International Journal of Molecular Sciences*. 2018 Dec 19;19(12):4121.
- A.E. Abdulle**, G.F.H. Diercks, M. Feelisch, D.J. Mulder, & H. van Goor. The Role of Oxidative Stress in the Development of Systemic Sclerosis Related Vasculopathy. *Frontiers in Physiology*. 2018 Aug 24;9:1177.
- A.E. Abdulle**, M.E. de Koning, H.J. van der Horn, M.E. Scheenen, G. Roks, G. Hageman, J. Spikman, J. van der Naalt. Early predictors for long-term functional outcome after mild traumatic brain injury in frail elderly patients. *Journal of Head Trauma Rehabilitation*. 2018 Nov/Dec;33(6):E59-E67.
- A.E. Abdulle**, J. van der Naalt. The role of mood, post-traumatic stress, post-concussive symptoms and coping on outcome after MTBI in elderly patients. *International review of psychiatry*. 2020 Feb;32(1):3-11.
- A.R. Bourgonje, **A.E. Abdulle** (shared first authorship), W. Timens, J.L. Hillebrands, G.J. Navis, S.J. Gordijn, M.C. Bolling, G. Dijkstra, A.A. Voors, A.D.M.E. Osterhaus, P.H.J. van der Voort, D.J. Mulder, H. van Goor. Angiotensin-converting enzyme 2 (ACE2), SARS-CoV-2 and the pathophysiology of coronavirus disease 2019 (COVID-19). *Journal of Pathology*. 2020 Jul;251(3):228-248.
- A.R. Bourgonje, **A.E. Abdulle** (shared first authorship), A.M. Al-Rawas, M. Al-Maqbali, M. Al-Saleh, M.B. Enriquez, S. Al-Siyabi, K. Al-Hashmi, I. Al-Lawati, M.L.C. Bulthuis, D.J. Mulder, S.J. Gordijn, H. van Goor, J. Saleh. Systemic Oxidative Stress Is Increased in Postmenopausal Women and Independently Associates with Homocysteine Levels. *International Journal of Molecular Sciences*. 2020 Jan;21(1):314.
- A.M. van Roon, **A.E. Abdulle**, D.J. Mulder. (2020). Pulse Wave Velocity in Systemic Sclerosis: potential beneficial effects of bosentan on forearm arterial stiffness? An exploratory study. *The Journal of Rheumatology*. 2021 Jan;48(1):154-156.
- P.R. van Dijk, **A.E. Abdulle**, M.L.C. Bulthuis, F.G. Perton, M.A. Connelly, H. van Goor, R.P.F. Dullaart. The Systemic Redox Status Is Maintained in Non-Smoking Type 2 Diabetic Subjects Without Cardiovascular Disease: Association with Elevated Triglycerides and Large VLDL. *Journal of Clinical Medicine*. 2019 Dec;9(1):49.
- A.M. van Roon, **A.E. Abdulle**, D. Zhang, A.J. Stel, M. Kuijpers, D.J. Mulder. Sympathicotomy in patients with drug resistant Raynaud's phenomenon. *Nederlandse Tijdschrift voor Geneeskunde*. 2021 Jun;165:D5630.
- T. Damba, A.R. Bourgonje, **A.E. Abdulle**, A. Pasch, S. Sydor, E.H. van den Berg, R.T. Gansevoort, S.J.L. Bakker, H. Blokzijl, R.P.F. Dullaart, H. van Goor, H. Moshage. Oxidative stress is associated with suspected non-alcoholic fatty liver disease and all-cause mortality in the general population. *Liver International*. 2020 Sep;40(9):2148-2159.
- M.F. Bourgonje, A.R. Bourgonje, **A.E. Abdulle**, L.M. Kieneker, S. la Bastide-van Gemert, R.T. Gansevoort, S.J.L. Bakker, D.J. Mulder, A. Pasch, J. Saleh, S.J. Gordijn, H. van Goor. Systemic oxidative stress, aging and the risk of cardiovascular events in the general female population. *Frontiers in Cardiovascular Medicine*. 2021 Feb;8:630543.

Bibliography

- A.M. van Roon, M. Kuijpers, S.C. van de Zande, **A.E. Abdulle**, A.M. van Roon, R. Bos, W. Bouma, T.J. Klinkenberg, H. Bootsma, M.J.L. DeJongste, M.A. Mariani, A.J. Smit, D.J. Mulder. Treatment of resistant Raynaud's phenomenon with single-port thoracoscopic sympathectomy: a novel minimally invasive endoscopic technique. *Rheumatology*. 2020 May;59(5):1021-1025.
- Z. Wang, A.R. Bourgonje, H. Groen, **A.E. Abdulle**, A.E.P. Cantineau, A.M. van Oers, L. van Dammen, M.L.C. Bulthuis, V. Wekker, B.W.J. Mol, T.J. Roseboom, H. van Goor, A. Hoek. *Journal of Clinical Medicine*. 2021 Sep;10(18):4243.
- J. Westra, E. Brouwer, E. Raveling-Eelsing, S. Arends, **A.E. Abdulle**, C. Roozendaal, M.A.M. van Delft, R.E.M. Toes, L.A. Trouw, A. Vissink, M.J. de Smit. Arthritis autoantibodies in individuals without rheumatoid arthritis: follow-up data from a Dutch population-based cohort (Lifelines). *Rheumatology*. 2021 Feb;60(2):658-666.
- P.R. van Dijk, A. Pasch, S.L. van Ockenburg-Brunet, F. Waanders, **A.E. Abdulle**, M.J. Muis, J.L. Hillebrands, H.J.G. Bilo, H. van Goor. Thiols as markers of redox status in type 1 diabetes mellitus. *Therapeutic advances in endocrinology and metabolism*. 2020 Feb; 11:2042018820903641.
- L.E. Van Eijk, M. Brinkhorst, A.R. Bourgonje, A.K. Offringa, D.J. Mulder, E.M. Bos, N. Kolundzic, **A.E. Abdulle**, P.H.J. van der Voort, M.G.M. Olde-Rikkert, J.G. van der Hoeven, W.F.A. van den Dunnen, J.L. Hillebrands, H. van Goor. Covid-19: immunopathology, pathophysiological mechanisms, and treatment options. *Journal of Pathology*. 2021 Jul;254 (4):307-331.
- M.H. Schoots, M.F. Bourgonje, A.R. Bourgonje, J.R. Prins, E.G.M. van Hoorn, **A.E. Abdulle**, A.C. Muller Kobold, M. van der Heide, J.L. Hillebrands, H. van Goor, S.J. Gordijn. Oxidative stress biomarkers in fetal growth restriction with and without preeclampsia. *Placenta*. 2021 Sep;115:87-96.