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A NOVEL PROPHYLACTIC STRATEGY TO MINIMIZE STEM CELL TRANSPLANTATION-RELATED COMPLICATIONS

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A Novel Prophylactic Strategy to Minimize Stem Cell Transplantation-related Complications

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my beloved family

献给我挚爱的家人

I will seek for the truth even though the journey is far and long.

路漫漫其修远兮，吾将上下而求索。

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ABSTRACT

Hematopoietic stem cell transplantation (HSCT) is a curative treatment and sometimes is the only option for patients with hematological malignancies and some types of solid tumors. It is also the first-line treatment for several severe genetic and metabolic disorders. The annual numbers of HSCT are continuously increasing, among which around 40% are allogeneic transplantations. However, the survival and life quality of those patients are largely compromised by transplantation-related complications, including acute graft-versus-host disease (aGvHD) and cardiovascular diseases. Oxidative stress is ubiquitously implied in the pathogenesis of these conditions and can thus serve as a treatment target. The present thesis provides a systematic investigation of a new prophylactic strategy employing antioxidants to combat HSCT-related complications.

Using a mouse model, we found that the onset of aGvHD is associated with intensive renal injury manifested by neutrophil gelatinase-associated lipocalin upregulation, hetero-lysosomes accumulation in the renal proximal tubular epithelial cells, and especially αKlotho depletion and increased circulatory FGF23. These findings suggest the important role of oxidative stress in the pathogenesis of aGvHD. Thereafter, two antioxidants N-acetylcysteine (NAC) and N-acetylcysteine amide (NACA) were investigated thoroughly in respect of their therapeutic potentials.

In order to compare the pharmacokinetic-pharmacodynamic properties and the mechanisms of action of NACA and NAC, we developed a new liquid chromatography-mass spectrometry method that could quantify both compounds in biological samples. We found that in relation to NAC, NACA has a higher oral bioavailability and superior capability to replenish glutathione.

In the aGvHD mouse model, NACA, but not NAC, was shown to ameliorate disease burden and prolong survival. Mechanistic studies demonstrated that the protective role of NACA was mediated by its antioxidative, anti-inflammatory and immuno-modulatory effects. Importantly, NACA did not negatively affect the engraftment efficiency in transplanted mice.

In addition to the prophylactic ability against aGvHD, NACA also exhibited a cardioprotective effect. We observed that NACA significantly attenuated the endothelial injuries induced by cyclophosphamide treatment. NACA preserved the integrity of the vascular endothelium *in vivo*, while reduced intracellular oxidative stress and cell death *in vitro*. Moreover, NACA assisted in maintaining the endothelial hemostasis and angiogenesis. Yet, NAC was relatively less efficient compared to NACA.

Taken together, the results obtained in the present thesis provide experimental evidence that the novel antioxidant, NACA, is a promising candidate for prophylaxis of HSCT-related complications. Further investigations are warranted to optimize the treatment regimen and to evaluate the influence of NACA on the clinical outcome of HSCT.

LIST OF SCIENTIFIC PAPERS

I. Risul Amin, **Rui He**, Dhanu Gupta, Mikhail Burmakin, Dara K. Mohammad, Joseph W. DePierre, Behnam Sadeghi, Hannes Olauson, Annika Wernerson, MD, Samir El-Andaloussi, Moustapha Hassan, Manuchehr Abedi-Valugerdi. The kidney injury casued by the onset of acute graft-versus-host disease is associated with down-regulation of αKlotho. *International Immunopharmacology*. 2020, 78: 106042.

II. **Rui He**, Wenyi Zheng, Tobias Ginman, Håkan Ottosson, Svante Norgren, Ying Zhao, Moustapha Hassan. Pharmacokinetic profile of N-acetylcysteine amide and its main metabolite in mice using new analytical method. *European Journal of Pharmaceutical Sciences.* 2020, 143: 105158.

III. **Rui He**, Wenyi Zheng, Kicky Rozing, Xiaoli Li, Yikai Yin, Weiying Zhou, Samir EL Andaloussi, Svante Norgren, Ying Zhao, Moustapha Hassan. The Role of N-acetylcysteine Amide in Acute Graft-versus-host Disease Mouse Model. (Submitted)

IV. **Rui He**, Wenyi Zheng, Terra Slof, Eva Wärdell, Agneta Månsson-Broberg, Svante Norgren, Ying Zhao, Moustapha Hassan. N-acetylcysteine Amide Alleviates Cyclophosphamide-induced Endothelial Damage. (Manuscript)

PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. Ying Zhao, **Rui He**, Sandra Oerther, Weiying Zhou, Massoud Vosough. Cardiovascular Complications in Hematopoietic Stem Cell Transplanted Patients. Journal of Personalized Medicine. 2022, 12: 1797.

II. Wenyi Zheng, **Rui He**, Xiuming Liang, Samantha Roudi, Jeremy Bost, Pierre-Michael Coly, Guillaume van Niel, Samir EL Andaloussi. Cell-specific targeting of extracellular vesicles though engineering the glycocalyx. *Journal of Extracellular Vesicles.* 2022, 11: e12290.

III. Esther Schoutrop#, Ibrahim El-Serafi#, Thomas Poiret#, Ying Zhao, Okan Gultekin, **Rui He**, Lidia Moyano-Galceran, Joseph W Carlson, Kaisa Lehti, Moustapha Hassan#, Isabelle Magalhaes#, Jonas Mattsson#. Mesothelin-Specific CAR T Cells Target Ovarian Cancer. *Cancer research.* 2021, 81: 3022-3035.

IV. Ahmed El-Serafi#, **Rui He#**, Wenyi Zheng, Fadwa Benkossou, Sandra Oerther, Ying Zhao, Karin Mellgren, Britt Gustafsson, Carsten Heilmann, Jukka Kanerva, Kourosh Lotfi, Jacek Toporski, Mikael Sundin, Martin Höglund, Jonas Mattsson, Ibrahim El-Serafi, Moustapha Hassan. Vitamin D levels and busulphan kinetics in patients undergoing hematopoietic stem cell transplantation, a multicenter study. Bone Marrow Transplantation. 2021, 56: 807-817.

V. Ying Zhao, Fei Ye, Torkel B Brismar, Xuan Li, **Rui He**, Rainer Heuchel, Ramy El-Sayed, Neus Feliu, Wenyi Zheng, Sandra Oerther, Joydeep Dutta, Wolfgang J Parak, Mamoun Muhammed, Moustapha Hassan. Multimodal Imaging of Pancreatic Ductal Adenocarcinoma Using Multifunctional Nanoparticles as Contrast Agents. *ACS Applied Materials & Interfaces*. 2020, 12: 53665-53681.

#: Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

HSCT	Hematopoietic stem cell transplantation
BMT	Bone marrow transplantation
EBMT	European Society for Blood and Marrow Transplantation
CIBMTR	Center for International Blood & Marrow Transplant Research
HLA	Human leukocyte antigen
AML	Acute myeloid leukemia
Cy	Cyclophosphamide
Bu	Busulphan
TBI	Total body irradiation
UCB	Umbilical cord blood
GvHD	Graft-versus-host disease
aGvHD	Acute graft-versus-host disease
cGvHD	Chronic graft-versus-host disease
TNFα	Tumor necrosis factor α
IFNγ	Interferon γ
IL	Interleukin
CVD	Cardiovascular diseases
CHF	Congestive heart failure
PTCy	Posttransplant cyclophosphamide
MMF	Mycophenolate mofetil
CNI	Calcineurin inhibitor
AKI	Acute kidney injury
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Nrf2	Nuclear factor erythroid 2-related factor 2
FGF-23	Fibroblast growth factor 23
CKD	Chronic kidney disease
NACA	N-acetylcysteine amide
NAC	N-acetylcysteine
GSH	Glutathione

PK	Pharmacokinetic
TCEP	Tris(2-carboxyethyl)phosphine
MPOZ	2-(methylsulfonyl)-5-phenyl-1,3,4-oxadiazole
MAF	Mafosfamide
HAECs	Human aortic endothelial cells
HUVECs	Human umbilical endothelial cells
PAS	Periodic acid-Schiff base
PASM	Periodic acid-Schiff methenamine
H&E	Hematoxylin and eosin
ICAM-1	Intercellular adhesion molecule 1
LC-MS	Liquid chromatography-mass spectrametery
NGAL	Neutrophil gelatinase-associated lipocalin
eNOS	Endothelial nitric oxide synthase
RT	Room temperature
LPAM-1	Integrin α4β7
BME	Basement membrane extract
PTECs	Proximal tubule epithelial cells
TEM	Transmission electron microscopy
AUC	Area under the curve
4-OH-Cy	4-hydroxycyclophosphamide
NO	Nitric oxide
MHC	Major histocompatibility complex
BH4	Tetrahydrobiopterin

1 INTRODUCTION

1.1 CANCER

Cancer is a group of diseases characterized by uncontrolled growth of abnormal cells with the ability to spread and invade adjoining tissues and metastasize throughout the body. As the second-leading cause of death, cancer contributed to almost 10 million deaths in 2020 worldwide (1). Cancer can be classified by either the histological type of tissue where it originates or the primary site where it first developed (2). Aiming to completely eradicate diagnosed cancer and prevent the relapse and metastasis of primary tumor, treatment options of cancer consist of surgery, radiotherapy, chemotherapy, targeted/biological therapy, and hormonal therapy.

1.2 HEMATOPOIETIC STEM CELL TRANSPLANTATION

Hematopoietic stem cell transplantation (HSCT), also known as bone marrow transplantation (BMT), is a combination of biological treatment, chemotherapy, or/and radiotherapy. HSCT was firstly performed in 1957 by Edward Donnall Thomas (3) and is currently a well-established curative treatment for patients with cancer including hematologic malignancies (e.g., leukemia, lymphomas, myelofibrosis, myeloma, and myelodysplasia) and some types of solid tumors (e.g., neuroblastoma, Ewing's sarcoma, and germ cell tumor) (4). It is also the only therapeutic modality for some severe nonmalignant disorders and genetic disorders, such as bone marrow failure, thalassemia, aplastic anemia, sickle cell disease, immune deficiency, and metabolic disorders. It has been assumed that 1.5 million HSCTs were performed from 1957 to 2019, with a yearly increment of 7% worldwide (5). According to the activity report from European Society for Blood and Marrow Transplantation (EBMT) involving 690 centers from 50 countries in Europe, Asia and Africa, a total of 45,364 transplants were taken place in 2020, and Sweden is one of the countries with highest frequency (Figure 1A and B) (6).

1.2.1 Classification

Based on the donor type, HSCT could be categorized as autologous or allogeneic transplantation, and the choice is dependent on donor availability and the underlying disease.

Autologous transplantation is still slightly more common than allogeneic. In the autologous setting, the patient is his/her own donor. Briefly, stem cells are harvested from the patient in remission and cryopreserved immediately. After intensive chemotherapy with or without radiotherapy, healthy stem cells are infused back to the patient. As reported by EBMT, the number of autologous transplants performed in Europe and collaborating countries in 2020 was 26,568, constituting 59% of the total transplantation cases. The leading indications for autologous transplantation were plasma cell disorder, non-Hodgkin's lymphoma and Hodgkin's lymphoma (Figure 1C) (6).

In the allogeneic setting, the stem cells are usually collected from:

- A human leukocyte antigen (HLA) matched family member;
- A HLA matched unrelated donor;
- A HLA half-matched family member, including parents, siblings or children (also called haplo-identical);
- An identical twin of the same parents (defined as syngeneic).

EBMT summarized that 18,796 allogeneic transplants were performed in 2020, accounting for the rest 41% of the total HSCTs (6). The frequency is close to that obtained from Center for International Blood & Marrow Transplant Research (CIBMTR) in the United States (7). The main indications for allogeneic transplantation are acute myeloid leukemia (AML), acute lymphoblastic leukemia, and myelodysplastic syndromes (Figure 1D) (6).

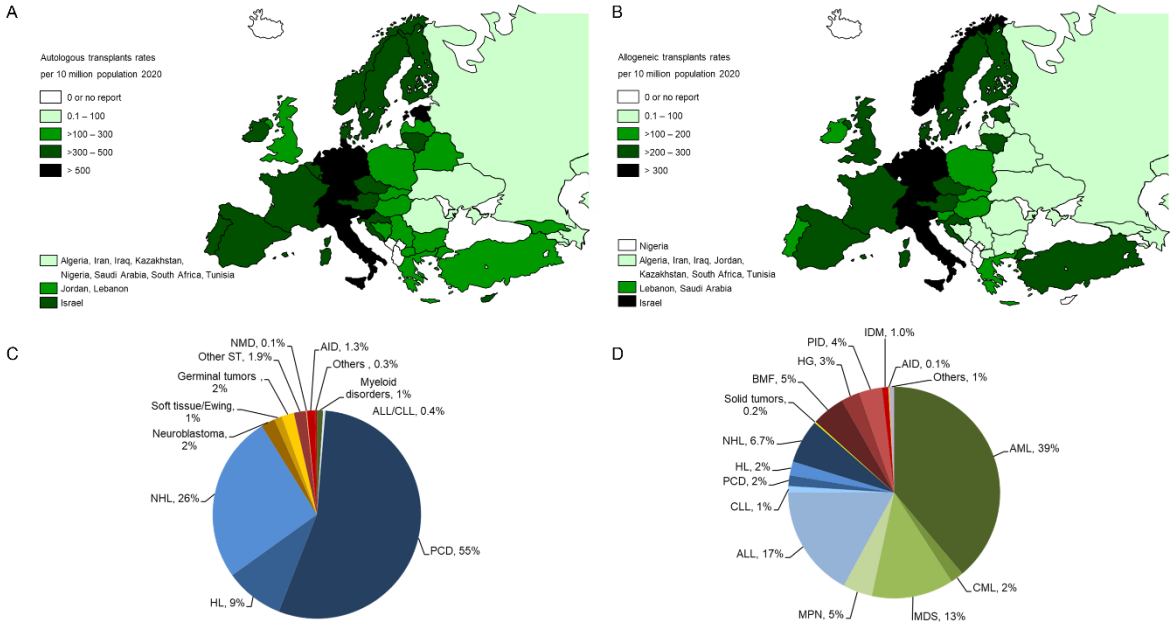

Figure 1. The incidences and indications of HSCT in Europe in 2020

Autologous (A) and allogeneic (B) transplant rate per 10 million population. Darker color represents for higher rate. (C-D) Pie charts showing the relative frequencies of disease indications for autologous (C) and allogeneic (D) HSCT. NHL: non-Hodgkin's lymphoma; HL: Hodgkin's lymphoma; PCD: plasma cell disorder; ALL: acute lymphoblastic leukemia; CLL: chronic lymphocytic leukemia; AID: auto-immune disease; MPN: myeloproliferative neoplasms; MDS: myelodysplastic syndromes; CML: chronic myeloid leukemia; AML: acute myeloid leukemia; IDM: inherited disorders of metabolism; PID: primary immunodeficiencies; HG: haemoglobinopathies; BMF: bone marrow failure.

1.2.2 Transplantation procedures

Basically, the HSCT procedure is constituted by three major phases including conditioning, stem cell infusion and post-transplant phase.

Prior to HSCT, patients are intensively treated/conditioned in order to eradicate diseased cells, suppress the host immune system, and provide a free space for donor cells, hence facilitating the engraftment. Traditionally, this has been achieved by myeloablative conditioning regimens, consisting of high doses of chemotherapy and/or irradiation. For instance, a combination of two or more chemotherapeutic drugs including cyclophosphamide (Cy), melphalan, busulphan (Bu), treosulfan, and fludarabine with or without total body irradiation (TBI) have been used in this setting. However, myeloablative conditioning is usually associated with intensive toxicities. To minimize severe side effects, reduced intensity and non-myeloablative conditioning regimens (e.g., fludarabine with lower doses of melphalan, Bu or treosulfan) have been introduced, making HSCT possible for the elderly and infirm individuals. Another driving factor for the application of reduced intensity and non-myeloablative conditioning is the recognition of graft-versus-tumor effects, i.e., immune reactions between donor cells and host malignant cells contributing to the success of HSCT (8).

Hematopoietic stem cells for transplantation can be harvested from bone marrow, peripheral blood, and umbilical cord blood (UCB). Compared to bone marrow-derived stem cells, those from peripheral blood are more frequently used due to faster engraftment (9). UCB stem cell transplantation is better suited for pediatric patients than adults, because only a small volume (approximately 80 mL (10)) of UCB could be gleaned. A recent study reported that UCB is a superior option to matched unrelated donor for pediatric AML patients when a matched sibling donor is not available, possessing better leukemia-free survival and less chronic graft-versus-host disease (cGvHD) (11) .

The post-transplant period is rationally divided into three phases: pre-engraftment (0-30 days), early post-engraftment phase (30-100 days), and late post-engraftment phase (>100 days) (12).

1.2.3 Cyclophosphamide

Cy is an alkylating agent that causes irreversible DNA crosslinking and programmed cell death. Thanks to the anti-neoplastic property, Cy has been prescribed in combination with other chemotherapeutic agents for the management and treatment of cancer for decades. For example, CHOP regimen (Cy, hydroxydaunorubicin, vincristine sulfate (Oncovin), and prednisone) is used as the standard treatment strategy for non-Hodgkin lymphoma (13), while TC (docetaxel and Cy) is usually applied to treat breast cancer (14).

Cy also has immunosuppressive potency with T-cell selectivity (15), thus it has been involved frequently not only in the conditioning phase prior to HSCT but also in the prevention of graft-versus-host disease (GvHD) post-transplantation. However, the doses of Cy used in HSCT-related applications are extremely high compared to its conventional dosage (900 mg/m^2 or 30

mg/kg), with a dose of >120 mg/kg administered over 2-4 days as conditioning (16), and a dose of 50 mg/kg/d given on days 3 and 4 after transplantation as prophylaxis (17).

1.3 COMPLICATIONS RELATED TO HEMATOPOIETIC STEM CELL TRANSPLANTATION

1.3.1 General complications

With the development in the advanced transplantation protocols, prophylaxis against severe infections, and optimized supportive care, the overall survival of patients undergoing HSCT has been improved significantly in recent years (18). However, post-transplant complications are still the main causes of morbidity and mortality apart from primary disease relapse. The risks and symptoms of the complications are associated with age, health status, comorbidities, applied conditioning regimen, and the type of transplantation. Depending on the time of onset, such adverse effects can be classified into early and late-stage complications (Table 1) (19, 20), where 100 days post transplantation is taken as the circumscription. Many of the early complications are related to conditioning regimen, while those appearing years after transplantation warrant lifelong surveillance. The prevalence of each side effect varies between adult and pediatric patients. For instance, the mortality caused by GvHD is higher in transplanted adults (7, 21), while the pediatric cohort suffers more from infections, pulmonary insufficiency, cardiovascular diseases, and endocrine disorders (22).

Table 1 Complications after hematopoietic stem cells transplantation

Early (usually ≤ 100 days post HSCT)	Late (usually > 100 days post HSCT)
Infections: bacterial, fungal, herpes simplex virus, cytomegalovirus	Infections: encapsulated bacteria, varicella-zoster, cytomegalovirus, pneumocystis pneumonia
Mucositis	Secondary malignancy
Sinusoidal obstructive syndrome/Veno-occlusive disease	Ocular: cataract
Pulmonary: idiopathic pulmonary syndrome, diffused alveolar hemorrhage, engraftment syndrome	Pulmonary: obstructive pneumonitis
Acute graft-versus-host disease (aGvHD)	Chronic graft-versus-host disease
Cardiovascular: cardiac failure	Cardiovascular: cardiomyopathy, hypertension
Hemorrhagic cystitis	Endocrine: hypothyroidism, infertility, growth failure for pediatric patients
Others: thrombotic microangiopathy, graft failure, graft rejection	Others: musculoskeletal, central nervous system, and renal disorders

1.3.2 Graft-versus-host disease

GvHD is the major complication related to allogenic HSCT, resulting in 10-20% of the mortality in the patients undergoing HLA-matched sibling and unrelated donor HSCT (23). GvHD is an immunological reaction, and its pathogenesis encompasses three consecutive steps: conditioning-derived inflammation; donor T-cell priming, expansion and differentiation; and target tissue destruction (24). Based on the time of onset, GvHD is categorized as acute and chronic (Table 1). Two additional subtypes of GvHD have been proposed at the National Institutes of Health Consensus Conference (25): late aGvHD that refers to the aGvHD occurring after 100 days; "overlap syndrome" that defines the simultaneous presence of both acute and chronic GvHD (26).

aGvHD typically occurs within 100 days post transplantation and mainly affects skin, liver, and gut. The grading of aGvHD severity is based on the tissue manifestations (Table 2) (27, 28). The highest GvHD grade that patients present in the first 100 days is correlated to their survival profile (29). A retrospective multicenter study including 126,838 adult patients between 1990 and 2015 reported an aggregate cumulative prevalence of 28% in 2010-2015 although the incidence of aGvHD decreased by time (30).

Table 2 Clinical stages and grades of aGvHD

Stage	Skin	Liver (bilirubin)	Gut
1	Rash < 25% of body surface	34-50 μmol/L	500-999 mL diarrhea/d
2	Rash 25-50% of body surface	51-102 μmol/L	1000-1500 mL diarrhea/d
3	Generalized erythroderma	103-225 μmol/L	> 1500 mL diarrhea/d
4	Bullous and desquamation	> 225 μmol/L	Severe abdominal pain
Grade	**Skin stage**	**Live stage**	**Gut stage**
I	1-2	0	0
II	1-3	1	1
III	2-3	2-4	2-3
IV	2-4	2-4	2-4

aGvHD was initially described as a "cytokine storm", since tissue and organ injury is mediated by cellular effectors and cytokines (24). Donor T cells play an essential role in aGvHD. On one hand, T cells are activated in response to allo-antigens and attack target tissues. Severe tissue inflammation recruits other effector immunological cells, including natural killer cells and neutrophils, to further amplify tissue damage (31). On the other hand, proliferation and differentiation of donor T cells is followed by a systemic elevation of Th1, Th17, and sometimes Th2-derived cytokines. These pro-inflammatory cytokines like tumor necrosis factor α (TNFα), interferon γ (IFNγ), interleukin 6 (IL-6), IL-1, IL-17 and IL-23 usually predominate the process of aGvHD.

cGvHD is the leading cause of late non-relapse death and occurs in 40-70% of the patients after allogeneic HSCT (32, 33). In contrast to aGvHD, cGvHD resembles the characteristics of

autoimmune vascular diseases and fibrosis. In response to the stimulation of donor T cells, donor B cells produce antibodies that accumulate in target tissues and contribute to the pathogenesis of cGvHD (34-36).

1.3.3 Cardiovascular diseases

Similar to GvHD, cardiac and vascular complications may emerge at early and late stages post HSCT. To minimize the incidence of cardiovascular diseases (CVD), prototypical risk factors like old age, conditioning regimen (e.g., radiotherapy and Cy), history or presenting cardiac disorders, and previously used drug (particularly anthracyclines and tyrosine kinase inhibitors) should be taken into consideration before making a transplant decision (37).

Acute cardiovascular complications including arrhythmias, pericardial effusion, cardiac tamponade, and congestive heart failure (CHF) may take place in short-term after HSCT. The prevalence of arrhythmias ranges from 9% to 27% in patients receiving HSCT (38); while the incidence of pericardial effusion is lower, with an incidence of 4.4-19% in pediatric cohorts (39). A retrospective study revealed that 2.3% of patients developed CHF within 100 days post allogeneic transplantation (40).

For the long-term survivors of HSCT, the risk of developing CVD increases over time post transplantation. In comparison to the general population, the risk of CVD-induced mortality is 2-4-fold higher in HSCT survivors (41). One study reported that the ten-year cumulative incidence of cardiovascular risk factors involving diabetes, hypertension, and dyslipidemia was 18.1%, 37.7%, and 46.7%, respectively (42). As summarized in a retrospective single-center study (43), these three risk factors were related to an increased rate of arterial events (e.g., coronary or peripheral artery disease), with a cumulative incidence of 1.5% at 5 years, 4.1% at 10 years, 12.8% at 20 years, and 22% at 25 years post HSCT. Coincidentally, both studies revealed that the prevalence was higher in older patients and those who underwent allogeneic HSCT.

Cy was found to be associated with the occurrence of cardiac events regardless of the time of exposure. When Cy was included in the conditioning regimen and given at a dose of 50 mg/kg/d for 4 days, 17% of the patients had symptoms and signs of CHF within 10 days post administration (44). Moreover, a recent study demonstrated that the cumulative incidence of early cardiac event was tripled in patients with posttransplant Cy (PTCy) treatment compared to those without the treatment (19% *vs* 6%) (45).

1.4 MANAGEMENTS OF HEMATOPOIETIC STEM CELL TRANSPLANTATION-RELATED SIDE EFFECTS

1.4.1 Symptomatic treatments

Acute graft-versus-host disease

Treatment of GvHD is usually tailored according to disease severity, presenting symptoms and clinical response. The first-line treatment for aGvHD is corticosteroids. Grade I aGvHD usually has a good response to local application with steroid, while systemic administration is needed for Grade II-IV aGvHD. However, long-term or high dose of corticosteroids treatment is sometimes associated with adverse effects. A randomized trial demonstrated that steroid treatment prevents Grade I aGvHD from escalating to Grade II, but the treated patients are at increased risk of infection (46). Furthermore, Lee *et al* (47) reported in another clinical study that treating Grade II-IV aGvHD with high-dose of steroids ($\geq$ 2 mg/kg of methylprednisolone) is related to steroid myopathy, which was identified in 41% of the patients.

In addition to the potential toxicity, up to 50% of patients do not respond significantly to corticosteroids treatment and have a poor prognosis in general (48). For steroid-refractory aGvHD, the most frequently used treatment strategies include anti-thymocyte globulin (49), TNFα antagonist (e.g., infliximab and etanercept) (50-52), IL-2 receptor inhibitor (53), mycophenolate mofetil (MMF) (54), extracorporeal photopheresis (55, 56), mesenchymal stem cells (57), and anti-metabolic agents (58). However, no evidence is available from multicenter, randomized clinical trials to show the efficacy of the aforementioned strategies, and considerable toxicity exists, such as infections, cytopenia, and nausea.

No consensus has been reached with regard to the management of steroid-refractory aGvHD until ruxolitinib was approved as the second-line treatment for aGvHD by FDA in 2019 (59). Ruxolitinib is a selective Janus kinase1/2 inhibitor with the ability to inhibit neutrophil migration, T-cell priming and expansion. A prospective, single-arm, phase II study reported the overall response in patients with steroid-refractory aGvHD to ruxolitinib was 54.9% (60). More recently, a multicenter, randomized study revealed a clear advantage of ruxolitinib in efficacy outcomes when compared to the best available therapy (61).

Cardiovascular diseases

Unfortunately, there is no specific guideline for managing HSCT-derived CVD. Like the treatment approaches in the general population, the therapeutic strategy for CVD in transplanted patients is chosen based on symptoms and diagnosis. For instance, systolic dysfunction, cardiomyopathy, and CHF are usually treated with angiotensin-converting enzyme inhibitors and beta blockers (62, 63), while arrhythmias are treated with beta blockers and sometimes calcium channel blockers (64).

1.4.2 Prophylactic treatments

Acute graft-versus-host disease

In contrast to symptomatic therapy, prophylactic treatment with the feature to prevent patients from suffering and reduce the use of health-care resources seems to be more advantageous. Since aGvHD is initially mediated by effector T cells, prophylactic regimens of aGvHD are still centered by calcineurin inhibitor (CNI, i.e., cyclosporine or tacrolimus) due to its ability to inhibit T-cell proliferation and activation. Other strategies (Figure 2) have been subsequently combined with CNI to improve efficacy and reduce the potential side effects (65, 66).

Methotrexate (67) and MMF are classic agents used for aGvHD prophylaxis owing to their anti-proliferative effect on donor T cells. The combination of CNI and methotrexate is typically applied for HLA-identical sibling transplants, while the MMF/CNI combination is more favorable in non-myeloablative conditioning and UCB-based transplant (68). Sirolimus, a mammalian target of rapamycin complex (mTOR) that can inhibit activation of both T cells and B cells, also has good prophylactic ability. A multicenter randomized phase III trial showed that adding sirolimus to the standard cyclosporine-MMF regimen significantly improved clinical outcomes (69). Another study, however, reported that sirolimus is related to higher incidence of veno-occlusive disease by inhibiting endothelial function after myeloablative HSCT, particularly when Bu is included in the conditioning regimen (70).

PTCy alone or together with other immunosuppressive substances is also used for GvHD prevention (71, 72) through inducing apoptosis of donor allo-activated T cells (73, 74). A recent study conducted by Battipaglia *et al* (75) demonstrated that AML patients who were treated with PTCy after 9/10 mismatched unrelated donor transplantation had a lower incidence of aGvHD compared to those who were treated with anti-thymocyte globulin. Furthermore, the former group showed a better leukemia/relapse/GvHD-free survival. Similar results were obtained in a study comparing PTCy-based regimen with cyclosporine-methotrexate regimen (76).

Another alternative approach is to deplete T cells *ex vivo* from the door pool, together with further immune modulation. This could be achieved by either $CD34^+$ selection (77) or CD3, α/β T cells depletion (78, 79). Recently, with recognition of the link between GvHD and gut microbiota, strategies of manipulating intestinal microbiota have been developed to prevent the emergence of GvHD (80). In addition, maraviroc (81), bortezomib (82), vorinostat (83), sitagliptin (84), and mesenchymal stem cells (85) are also under investigation for GvHD prophylaxis.

Importantly, based on tremendous convincing preclinical and clinical data, the co-stimulation blockade agent abatacept was approved by FDA in 2021 for prevention of aGvHD in combination with a CNI and methotrexate in HSCT patients (from a matched or 1 allele-mismatched unrelated-donor) aged $\geq$ 2 years old. However, albeit with superior clinical

effectiveness, abatacept is also associated with a number of adverse reactions, such as anemia, hypertension, infection, and acute kidney injury (AKI) (86).

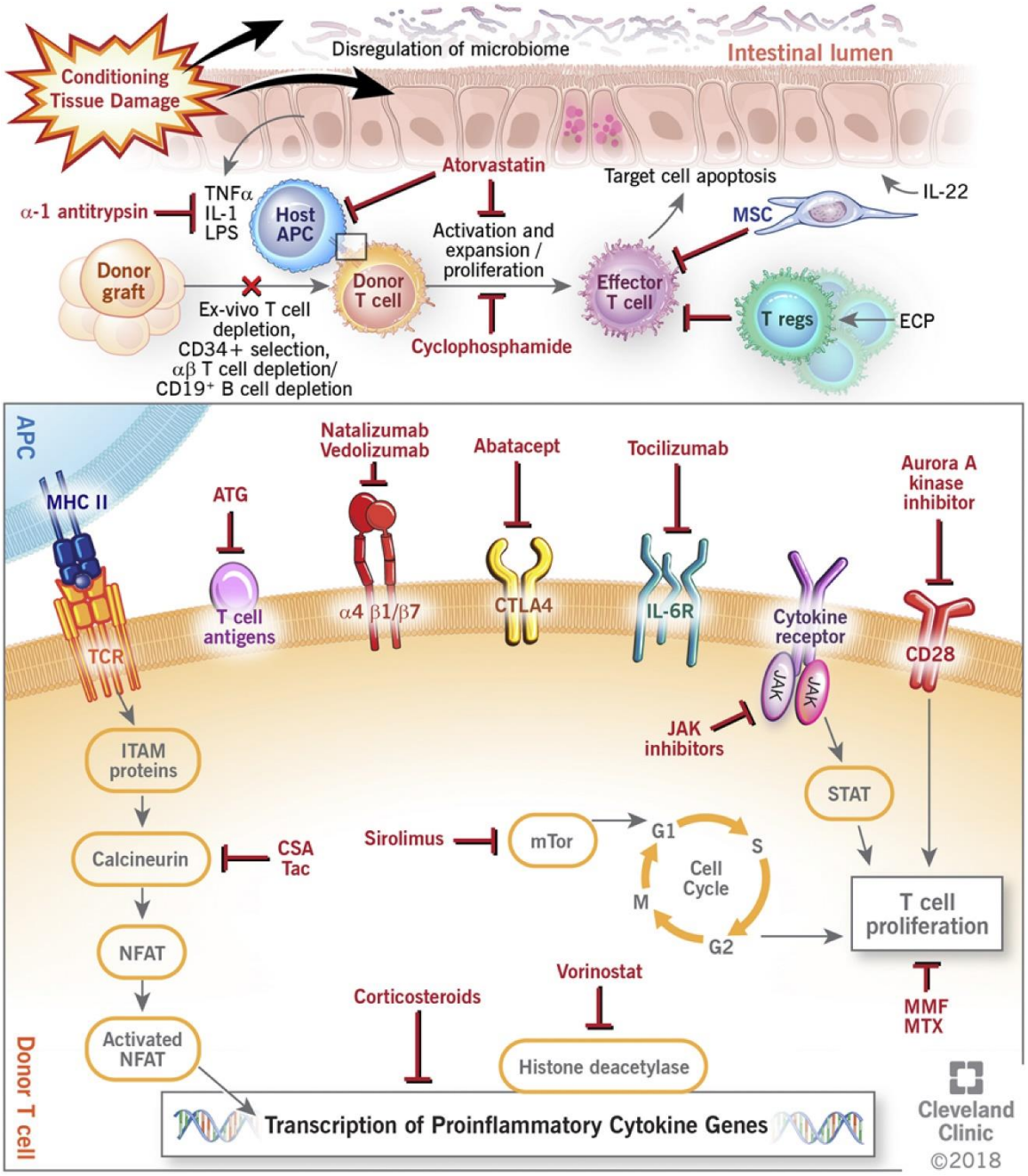

Figure 2. Current approaches to prevent and treat GvHD

LPS: lipopolysaccharides; APC: antigen-presenting cell; MSC: mesenchymal stem cells; Treg, regulatory T cell; ECP: extracorporeal photopheresis; MHC II: major histocompatibility complex II; TCR, T-cell receptor; ATG: anti-thymocyte globulin; CTLA4: cytotoxic T lymphocyte antigen 4; JAK: janus kinase; ITAM: immunoreceptor tyrosine-based activation motif; CSA: cyclosporine; Tac: tacrolimus; NFAT: nuclear factor of activated T cell; mTor: mammalian target of rapamycin complex; STAT: signal transducer and activator of transcription protein; MMF: mycophenolate mofetil; MTX: methotrexate.

Cardiovascular diseases

Since a broad range of risk factors are related to the development of CVD in HSCT, cautious follow-up with cardiovascular screening and comprehensive monitoring are warranted in the patients. Prior to HSCT, more attention should be paid to patients with older age, history of anthracycline use, and coexisting cardiac disorders, such as abnormal left ventricle function and heart failure. In the conditioning regimen, high doses of Cy, melphalan, fludarabine, and chest radiation should be used with caution or avoided. Maintenance strategies used after transplantation need to be assessed thoroughly as well. For example, sorafenib has been reported to reduce disease relapse and improve overall survival in transplanted patients, but it was also found to cause left ventricular systolic dysfunction (87, 88).

Other practices are also recommended by the Center of International Blood and Marrow Transplantation, including EBMT and CIBMTR, for mitigating the potential cardiovascular risk factors in HSCT survivors (89). A full clinical examination should be performed yearly post transplantation. In addition, fasting plasma glucose should be tested every 3 years after the age of 45 or if the patient has sustained high blood pressure. Dyslipidemia testing is suggested every 5 years for adult patients and every 2 years for pediatric patients. Moreover, implementation of cardiac imaging methods, including electrocardiogram, echocardiography, and cardiac magnetic resonance imaging, are advocated to monitor and identify risk factors at an early stage. Furthermore, cardiovascular status could also benefit from appropriate lifestyle modifications and physical training.

1.4.3 Redox-based treatments

It has been broadly reported that elevated levels of reactive oxygen species (ROS), which can cause cellular excessive oxidative stress, are involved in the development and progression of aGvHD as well as cardiovascular complications (90-95). ROS are important byproducts of cell metabolism and can also be induced by radiotherapy and chemotherapy. Cellular ROS is counter-balanced by a serial of antioxidative enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. Following this rationale, substantial preclinical studies have been conducted to develop new prophylactic treatment for aGvHD and HSCT-related CVD through targeting the redox system, either by preventing ROS generation or to neutralize excessive ROS.

Acute graft-versus-host disease

Keon-Il Im and colleagues (96) exerted a free radical scavenger NecroX-7 to regulate aGvHD. Recipient mice were intravenously administered with NecroX-7 from day one after TBI to day 14 post BMT. Compared to control mice, the survival index of the treated group was significantly improved, with less severe aGvHD-related tissue damage. In a subsequent study, the antioxidative protein thioredoxin-1 was shown to inhibit donor T-cell allo-reaction and migration in aGvHD by reducing ROS (97). Studies have also reported that aGvHD could be

mitigated by other redox-based strategies, such as administration of NADPH oxidase inhibitor apocynin (98) and transplanting with Sirtuin 3-knockout T cells (99).

Conflicting findings were reported when nuclear factor erythroid 2-related factor 2 (Nrf2), the redox master regulator, was served as the target for aGvHD management. Dimethyl fumarate and sulforaphane ameliorated aGvHD via activating Nrf2 pathway (100, 101). Oppositely, transplantation with Nrf2-depleted T-cell decreased aGvHD-derived morbidity and mortality (102).

Cardiovascular diseases

Oxidative stress-mediated cardiotoxicity in HSCT patients is highly correlated with conditioning regimens, co-administered drugs, and pro-inflammatory cytokines. ROS accumulation can damage endothelial cells and cardiomyocytes that are fundamental components of cardiovascular system. For Cy-induced cardiotoxicity, a serial of natural antioxidants, lipid acid, selenium compounds, nicorandil, and allopurinol showed promising protective effects in preclinical studies (103-107). Unfortunately, there is a lack of compelling evidence supporting translational potentials of these strategies. In addition, limited investigations are available regarding redox-based treatment for other CVD risk factors.

1.5 KLOTHO

The *Klotho* gene was discovered and identified as an anti-aging gene in 1997 (108) and consists of three subfamilies: *αklotho*, *βklotho*, and *γklotho*. αKlotho is a transmembrane protein encoded by the *αklotho* gene and abundantly expressed in kidney and brain (109). The membrane-bound αklotho acts as a cofactor for fibroblast growth factor 23 (FGF-23) and FGF receptors to increase their binding affinity (110), thereby taking part in the regulation of vitamin D biosynthesis and calcium and phosphate homeostasis (111). The extracellular domain of αKlotho can be cleaved by membrane proteases and shed into the systemic circulation (112).

1.5.1 FGF23-αKlotho axis in inflammation

FGF23-αKlotho axis is involved in the development of several chronic disorders, not only via modulating mineral metabolism, but also through regulating inflammation. The most well-known manifestation where the FGF23-αKlotho axis is impaired is chronic kidney disease (CKD). At the early stage of CKD, αKlotho declines in serum and urine followed by an increased level of FGF23 in serum (113). Excess FGF23 influences the immune system by inhibiting neutrophils activation, reducing leukocyte recruitment, and promoting pro-inflammatory cytokine releasement (114). FGF23-αKlotho axis dysfunction is also implicated in other chronic inflammatory diseases, such as inflammatory bowel disease and chronic obstructive pulmonary disease (115, 116).

1.5.2 αKlotho and oxidative stress

Given that aging and CKD are associated with oxidative stress and αKlotho downregulation, the interactions between αKlotho and redox hemostasis have been studied. Nagai *et al* (117) found that the antioxidant a-tocopherol reversed the aging-associated cognition impairment in *Klotho*-mutant mice. Moreover, Klotho overexpression protected dopaminergic neurons against oxidative stress in transgenic mice, partially by modulating the activation of apoptosis signal-regulating kinase 1/p38 mitogen-activated protein kinases (118). In rat aortic smooth muscle cells, delivery of *Klotho* gene suppressed NADPH oxidase 2 expression and oxidative stress (119).

1.6 N-ACETYLCYSTEINE AND N-ACETYLCYSTEINE AMIDE

1.6.1 N-acetylcysteine

Supplementation with exogenous antioxidants is widely adopted to prevent and treat oxidative stress-induced disorders. Among all antioxidants, multifunctional N-acetylcysteine (NAC) stands out with its ability to replenish glutathione (GSH) synthesis as well as to scavenge ROS. NAC was approved by FDA in 1963 as a mucolytic agent (120) and is also used clinically as an antidote for paracetamol overdose.

Substantial studies are available in support of NAC for managing oxidative stress-related pathologies. For example, pretreatment of NAC protected rats from folic acid-induced acute kidney disease and the underlying mechanisms included mitochondrial energy metabolism and redox homeostasis (121). Another investigation demonstrated the hepatic protective effect of NAC in patients receiving Bu as a part of the conditioning regimen in HSCT (122). Recently, NAC showed anti-inflammatory and immunomodulatory ability in ovalbumin-sensitized rats (123).

In particular, a very high dose of NAC is required to achieve therapeutic effect. One meta-analysis concluded that a dose of 600 mg/d, or even more (≥ 1,200 mg/d), is needed for the treatment of chronic bronchitis or chronic obstructive pulmonary disease (124). In a randomized controlled trial, Kiuru *et al* applied postnatal NAC in infants with extremely low birth weight for the purpose of neuroprotection, but failed to observe a long-term improvement (125). More importantly, controversial results about the effect of NAC on GvHD was reported (122, 126). The limiting factors for the abovementioned studies might involve generally infirm health condition, poorly matched basic characters in treatment arms, and inconsistent treatment durations of NAC. Another important factor may be ascribed to the hydrophilic properties (Figure 3) (127) that can impede the transmembrane transport of NAC in the targeted cells.

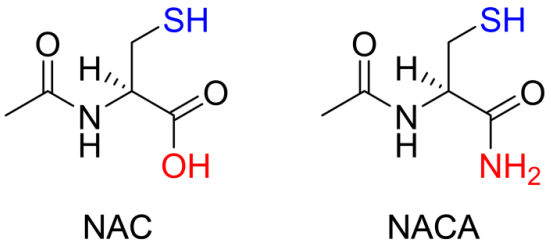

Figure 3. Chemical structures of NAC and NACA

NAC: N-acetylcysteine; NACA: N-acetylcysteine amide.

1.6.2 N-acetylcysteine amide

N-acetylcysteine amide (NACA) is the amide derivative of NAC (Figure 3). It was synthesized in 1967 conferring higher lipophilicity and cell-membrane permeability to improve the antioxidative effect of NAC. In recent years, several preclinical studies have indicated robust antioxidative capability and good tolerability of NACA *in vivo*.

Prophylactic administration of NACA reduced doxorubicin and trastuzumab-induced cardiac dysfunction in mice, by attenuating oxidative stress/cardiac apoptosis and increasing left ventricular ejection fraction (128). Post-blast treatment with NACA showed a significant protective effect on the blood-brain barrier integrity of experimental rats (129). Moreover, selenite-induced cataracts were reversed by intraperitoneal injection of NACA, with higher GSH level, m-calpain level and activity, and thioltransferase activity in rats (130). Furthermore, NACA was proved to be anti-inflammatory in neurodegenerative-related disorders (131). Collectively, it would be promising to investigate the role of NACA in HSCT-related complications in which oxidative stress and inflammation are involved.

1.6.3 Pharmacokinetics

To fully explore the therapeutic potency of NACA, its pharmacokinetic (PK) properties need to be characterized. However, there is only one study (132) about the tissue distribution of NACA after oral administration. It is important to conduct a comprehensive study to reveal its PK/pharmacodynamic (PD) behavior (i.e., absorption, distribution, metabolism, and elimination in relation to its effect).

Since NAC was reported to be a metabolite of NACA, it is a prerequisite to apply an analytical method which separates and quantifies both compounds simultaneously. However, all existing quantitative assays of NACA and NAC failed to achieve satisfactory separation and measurement, either due to overlap peaks (132) or because of inadequate extraction (133).

2 RESEARCH AIMS

2.1 GENERAL AIM

To explore the role of NACA in HSCT-related complications

2.2 SPECIFIC AIMS

- To characterize renal manifestations at the onset of aGvHD and to investigate the role of αKlotho during these manifestations
- To simultaneously quantify NACA and its metabolite NAC in plasma samples and to determine their PK and PD parameters
- To investigate the prophylactic effect of NACA on aGvHD in mice
- To elucidate the protective ability of NACA against Cy-induced endothelial dysfunction and to explore the underlying mechanism

3 MATERIALS AND METHODS

3.1 MATERIALS

Cy monohydrate (C0768), Bu (B2635), GSH (G6013), tris(2-carboxyethyl)phosphine (TCEP, C4706), N-ethylmaleimide (E3876), ammonium bicarbonate (NH_4CO_3, 09830), and NAC (A7250) were purchased from Sigma Aldrich (St. Louis, Missouri, USA), while NACA was provided by Dr. Glenn Goldstein (Sentient Life Sciences Inc, New York, USA). 2-(methylsulfonyl)-5-phenyl-1,3,4-oxadiazole (MPOZ, GN58362) and mafosfamide (MAF, D-17272) were bought from Aurum Pharmatech (New Jersey, USA) and Niomech-IIT GmbH (Bielefeld, Germany), respectively.

NACA and NAC were freshly dissolved in saline (25 mg/mL, for animal experiments) or cell culture medium (20 mM, for cell experiments) and neutralized with NaOH prior to use. Cy monohydrate was dissolved in saline at a concentration of 20 mg/mL, while MAF was prepared on ice at a concentration of 5 mM for further dilution. All stock solutions were sterilized using a 0.22 μm filter (Millipore, Merck) before application.

3.2 ANIMALS

Animal experiments were approved by the Stockholm Southern (S67-14, S1034-17) and Linköping Animal Research Ethical Committee (11257-2020) and performed according to the Swedish Legislation for Animal Welfare. Balb/c and C57bl/6 mice were purchased from Charles River and maintained in a standard pathogen-free facility with a temperature of 20 ± 2 °C, a relative humidity of 50 ± 5%, and 12 h dark/12 h light cycles. All mice had free access (*ad libitum*) to diet and water.

3.2.1 aGvHD mouse models

Two different aGvHD mouse models were established in the present thesis. In **Study I**, each recipient Balb/c mouse was conditioned with Bu (*i.p.*, 20 mg/kg, once daily, from day -7 to day -4) and Cy (*i.p.*, 100 mg/kg, once daily, from day -3 to day -2), and was transplanted via intravenously injection (day 0) with 30×10^6 bone morrow cells and 20×10^6 splenocytes from C57bl/6 mice in the allogeneic setting or from Balb/c mice in the syngeneic setting. In **Study III**, the conditioning phase was carried out on the recipients using TBI at a dose of 800 cGy (fractioned into two doses with a 6-h interval), followed by a transplantation with 5×10^6 bone marrow cells and 5×10^6 splenocytes from the donor. Recipient mice were then monitored daily and evaluated based on a well-established GvHD scoring system (134) including five parameters: body weight, fur texture, hunching posture, activity, and skin integrity. Oral antibiotics and subcutaneous saline administration were given as supportive treatments when necessary. NACA or NAC was given orally to the recipient mice at a dose of 250 mg/kg (*bid*) from day -1 to day 14 post transplantation as the prophylactic treatments.

3.2.2 Pharmacokinetic study

NACA or NAC was administered orally (*p.o.*) or intravenously (*i.v.*) at a dose of 300 mg/kg to female Balb/c mice. Blood samples were collected in EDTA-coated tubes at seven different time points post administration (0-180 min) and plasma was separated for further analysis. Using WinNonLin software (Standard edition, version 2.0), the concentration-time curves of NACA, NAC and GSH were modeled, and the PK parameters and bioavailability were calculated.

3.2.3 Cardiovascular toxicity mouse model

Cy was used to induce cardiovascular toxicity in Balb/c mice. Two groups of mice were pre-treated with NACA or NAC at a dose of 250 mg/kg (*bid*, *p.o.*) for seven days (day -7 to day -1), while the third group received an equal volume of saline instead. On day 0, Cy was injected (*i.p.*) into Balb/c mice at a single dose of 400 mg/kg. NACA, NAC or saline was administered to the mice for another 7 days (day 1 to day 7). After all treatments, mice were sacrificed, and aorta specimens were collected for further analysis.

3.3 CELL CULTURE AND TREATMENTS

Human aortic endothelial cells (HAECs, C-12271) and human umbilical endothelial cells (HUVECs, C-12203) were purchased from Promocell GmbH (Heidelberg, Germany). All cells were maintained at 37 °C with 5% CO_2 and cultured in endothelial cell media MV2 (Promocell, C22022) supplemented with penicillin-streptomycin (Sigma-Aldrich, P4333). Cells from passages 2-10 were used for experiments, except for tube formation assay, in which cells with passages 3-4 were utilized.

3.4 SERUM ANALYSIS

To quantify serum cytokines, blood samples were collected on day 4-5 (**Study I**) or on day 7 (**Study III**) after BMT. The levels of cytokines were measured by a mouse Th1/Th2 Cytokine Kit (BD Biosciences, 551287), IL-1 beta Mouse ELISA Kit (Invitrogen, BMS6002), and Mouse Th Cytokine Panel (BioLegend, 741043) in accordance with the manufacturers' protocols. Serum levels of FGF23 were determined by a commercial ELISA kit (Immunotopics International, 60-6300) according to the manufacturer's guidelines.

3.5 DETERMINATION OF OXIDATIVE STRESS

3.5.1 Reactive oxygen species

In **Study III**, intracellular ROS levels in splenocytes were examined by fluorescent staining using CellROX^{TM} Green (Invitrogene, C10448C) according to the manufacturer's instructions. Briefly, spleens from recipient mice were collected to prepare single-cell suspension. After permeabilization, cells were stained with 5 μM CellROX^{TM} Green and incubated at 37 °C for 30 min. The fluorescence intensity of each sample was analyzed by a MAQSQuant flow cytometer.

In **Study IV**, ROS levels in endothelial cells were evaluated by a fluorescence-based kit (Abcam, ab13851). In brief, HAECs or HUVECs were seeded in a black 96-well plate (8×10^3 cells/well) and pre-treated with NACA or NAC for 4 h. After wash, cells were then stained with DCF-DA for 45 min and incubated with 85 μM or 100 μM MAF for 3 h, respectively. Fluorescence intensities were measured using a microplate reader (SpectraMax i3x, Molecular devices).

3.5.2 Enzyme activities

Cellular catalase and total SOD activities were determined in HAECs and HUVECs. Cells were seeded in petri dishes (1×10^6 cells/dish) and treated with NACA or NAC for 6 h, followed by MAF treatment for 20 h. After harvesting, cells were lysed by sonication. Activities of enzymes were measured using commercial kits (Invitrogen, EIACATC and EIASODC) according to manufacturer's instructions and results were normalized with total protein concentration for each sample.

3.5.3 Cellular glutathione level

HAECs and HUVECs were treated as in section 3.5.2, harvested, and lysed with mammalian cell lysis buffer (Abcam, ab179835). To exclude the enzymes that can interfere with the assay, protein was precipitated using a deproteinizing sample preparation kit (Abcam, ab204708). Cellular reduced GSH levels were determined using a commercial assay (Abcam, ab205811) and the fluorescence of each sample was monitored at Ex/Em = 490/520 nm with a microplate reader.

3.6 HISTOLOGICAL ANALYSIS

Recipient mice were sacrificed on day 4 (**Study I**) or day 7 (**Study III**) after BMT. Kidney, skin, liver, and colon specimens were isolated, fixed, and embedded in paraffin. The kidney sections were stained with periodic acid-Schiff base (PAS) and periodic acid-Schiff methenamine (PASM), while the rest tissues were stained with hematoxylin and eosin (H&E) using standard protocols.

3.7 IMMUNOHISTOCHEMISTRY

Paraffin-embedded sections were rehydrated, and the antibodies were retrieved. After blocking, kidney and aorta sections were stained with a monoclonal anti-CD68 antibody (Dako, Denmark) and a recombinant anti-intercellular adhesion molecule 1 (ICAM-1) antibody (Abcam, ab179707), respectively. To visualize the antigens expression, horseradish peroxidase (HRP)-conjugated secondary antibodies and 3,3'-diaminobenzidine (DAB) peroxidase substrate kit (Abcam, ab64238) were applied successively.

3.8 *IN SITU* HYBRIDIZATION

In situ hybridization was performed on formalin-fixed and paraffin-embedded kidney sections. Probes against the base sequence (879-1844) of murine αKlotho mRNA (422087) were hybridized followed by RNAscope (Advanced Cell Diagnostics, USA) amplification based on the manufacturers' instruction. Red chromogenic dots were detected.

3.9 IMMUNOBLOTTING

Total protein concentration of tissue homogenates and cell lysates were quantified using Pierce™ BCA protein assay. Sample extracts were fractionated on 4-15% Mini protean® TGX™ 174 precast gels (Bio-Rad, 456-1085). After electro-transfer and blocking, membranes were incubated with following primary antibodies: anti-neutrophil gelatinase-associated lipocalin (NGAL, Santa Cruz Biotechnology, sc-515876), anti-αKlotho (TransGenic, KM2076), anti-pan-14-3-3 (Santa Cruz Biotechnology, sc-1657Ink), anti-actin (Sigma-Aldrich, A5441), anti-endothelial nitric oxide synthase (eNOS, Abcam, ab76198), anti-Arginase I (Invitrogen, PA5-85267), anti-Arginase II (Invitrogen, PA5-78820), anti-Jagged-1 (CST, 2620S), anti-HES-1 (ThermoFisher, PA5-28802), or anti-Notch-1 (CST, 3608S). Thereafter, fluorescent secondary antibodies (Li-cor, Germany) were applied, and the protein bands were visualized by Odyssey CLx system (Li-cor, Germany).

3.10 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Plasma samples from mice in section 3.2.2 were subjected to TCEP/MPOZ derivation. In brief, 10 µL plasma was mixed with 5 µL NH_4CO_3 (10 mM in H_2O), followed by serial addition of 40 µL MPOZ (0.045 M in dimethylacetamide) and 5 µL TCEP (0.2 M in H_2O). After reaction at room temperature (RT) for 30 min, the mixture was deproteinized by 100 µL acetonitrile. The supernatant was detected for NACA, NAC and GSH using the liquid chromatography-mass spectrometry (LC-MS) analyzer (Thermo TSQ Quantum Ultra). Briefly, 2 µL supernatant was injected to a LC system (Agilent 1100), separated by a C18 column and detected via MS. Detailed method establishment and validation were available in our publication (127).

3.11 FLOW CYTOMETRY

3.11.1 General analysis

Single-cell suspensions from recipient spleens were prepared and underwent surface and intracellular staining with fluorochrome-conjugated antibodies. Specifically, direct incubation with pre-mixed antibodies (30 min, 4 °C) was performed for surface staining, while cells were fixed and permeabilized before intracellular staining.

The following antibodies from BD Bioscience, BioLegend, or eBioscience were applied: Alexa Fluor 647 anti-H2k^{b} (562832), eFluor 450 anti-H2k^{b} (48-5958-82), PE anti-H2k^{d} (553566), PE Cy7 anti-CD4 (100528), FITC anti-CD8 (100706), APC anti-CD25 (102012), PE anti-Foxp3 (126404), APC anti-CXCR3 (126512), and PE anti-Integrin α4β7 (LPAM-1, 120605). To exclude the unspecific interference, corresponded isotype control staining was conducted. Data was collected using a MAQSQuant flow cytometer.

3.11.2 Intracellular cytokine measurement

To detect the intracellular cytokines, splenocytes from recipient mice were collected on day 7 post transplantation. Cells were then stimulated *in vitro* with Cell Activation Cocktail (BioLegend, 423304) at 37°C for 6 h, followed by fixation with a CytoFast Fix/Perm Buffer set (BioLegend, 426803). Cells were subsequently incubated with different combinations of the antibodies including PE conjugated anti-IL-4 (504104), anti-TNFα (506306), anti-IL-17A (12-7177-81); APC-conjugated anti-IL-10 (505010), anti-IL-2 (503810), anti-IFNγ (505810). All the antibodies were purchased from BioLgend or eBioscience. Stained cells were analyzed by a MAQSQuant flow cytometer.

3.11.3 *In vivo* proliferation

Splenocytes from donor (C57bl/6) mice were harvested and labelled with CellTrace^{TM} Violet (Invitrogen, C34557) according to manufacturer's instructions. After conditioned with TBI, recipient (Balb/c) mice were transplanted with 8×10^{6} of the labeled splenocytes. Three days after transplantation, recipients' spleens were isolated and the violet expression levels of CD4^{+} and CD8^{+} splenocytes were determined by a flow cytometer (Merck, CellStream^{TM}).

3.11.4 Cell apoptosis assay

HAECs and HUVECs were seeded in 6-well plates (2×10^{5} cells/well) and pre-treated with 1-5 mM of NACA or NAC for 6 h. Cells were harvested by gentle trypsinization. Cell apoptosis was measured using PE-Annexin V apoptosis detection kit I (BD, 559763) in accordance with manufacturer's instructions. Data were collected on a flow cytometer (FACS Array, BD).

3.11.5 DNA damage assay

HAECs and HUVECs were treated as mentioned above and were fixed with 80% methanol. After permeabilization with 0.1% Triton-X (10 min, RT), cells were blocked in 3% BSA (1 h, RT), followed by incubation with recombinant anti-γH2A.X (phospho S139) antibody (1:200,

Abcam, ab81299) overnight at 4°C. FITC-labelled secondary antibody (1:1000, Abcam, ab7086) was applied to detect expression levels of intracellular γH2A.X. Data was acquired using a flow cytometer (CellStreamTM, Merck).

3.12 ANGIOGENESIS ASSAY

Endothelial cell tube formation assay was performed using a PromoKine angiogenesis assay kit (PromoCell, PK-CA577-K905) according to manufacturer's protocol. Briefly, pre-thawed growth factor reduced basement membrane extract (BME) matrix was added into a pre-cooled 96-well plate. The plate was incubated at 37 °C for 1 h to polymerize BME gels. NACA or NAC pre-treated cells or non-treated (control) cells were suspended in MAF-containing MV2 medium, seeded onto the gels and incubated for 24 h at 37 °C. Tubular structures were captured by a phase-contrast microscope (Olympus, CellSens software 1.16) at 40× magnification.

3.13 DATA ANALYSIS AND STATISTICS

All flow cytometery data were analyzed using FlowJo software (v.10.6.2), except for the serum cytokine study where the results were analyzed by BD CellQuest™ software or LEGENDplex Data Analysis Software in accordance with the kits applied. Modeling of concentration-time curves and calculation of PK parameters (e.g., area under the curve (AUC) and half-time) were carried out utilizing WinNonLin software. Results from immunoblotting were processed using Image Studio Lite software (v.5.2.5). Tube formation images were analyzed with Angiogenesis analyzer in ImageJ software (v.2.0).

Statistical analysis was performed using GraphPad Prism software (v.8). The differences in survival between groups were evaluated by the long-rank test. Two-tailed Mann-Whitney test was used to compare the means between two groups. For all tests, p values < 0.05 were considered statistically significant.

4 RESULTS

4.1 KLOTHO WAS DOWNREGULATED IN THE ACUTE GVHD MOUSE MODEL

Renal injury and aGvHD are life-threatening complications which frequently occur following HSCT. aGvHD is also regarded as a risk factor of inflammatory kidney damage in some cases. The first aim of the current study was to elucidate the occurrence and extent of renal injury at the onset of aGvHD. Furthermore, since aKlotho is associated with kidney inflammation and has been reported to be deficient in the context of acute/chronic kidney injury (113, 135), our second aim was to figure out the alteration patten of aKlotho at the onset of aGvHD.

4.1.1 Emergence of renal injury at the onset of aGvHD

From approximately 4-7 days after allogeneic transplantation, the recipient mice began to show aGvHD symptoms. Compared to control and syngeneic-transplanted mice, allo-transplanted mice had obvious body weight loss, hunching position, decreased movement, ruffled fur texture and diarrhea, resulting in high clinical severity scores (Figure 4A). Thus, day 4 was considered as the onset of aGvHD. Successful transplantation was confirmed by chimeras in spleen and bone marrow. Serum levels of cytokines, including TNFα and IFNγ, were quantified due to their vital roles at the onset of aGvHD (136). Indeed, dramatic increments were observed for both cytokines in aGvHD mice in relation to their counterparts (Figure 4B and C). Importantly, the kidney damage biomarker NGAL was found to be significantly upregulated in kidneys of aGvHD mice (Figure 4D).

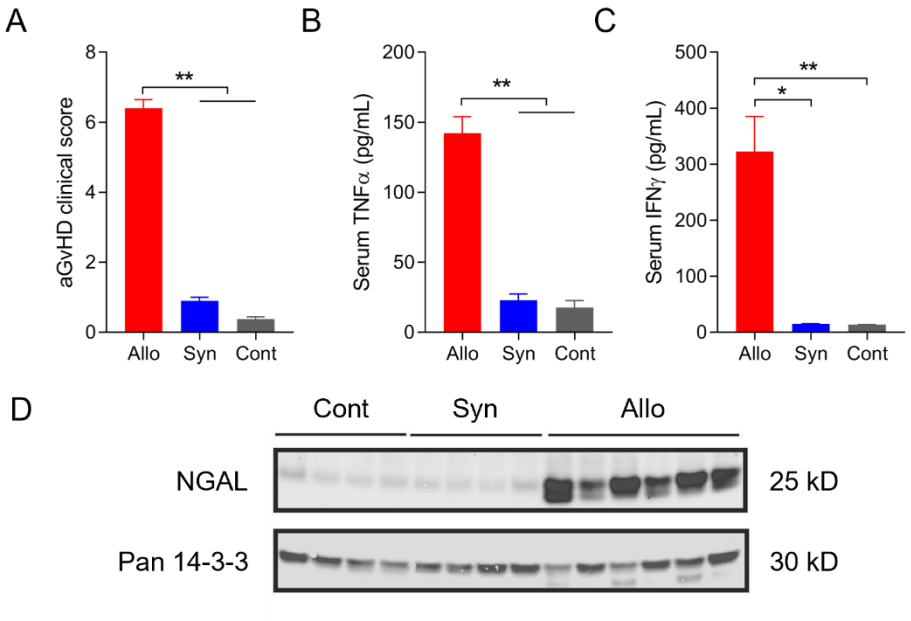

Figure 4. Kidney injury in aGvHD mouse

Balb/c mice were conditioned with Bu-Cy from day -7 to day -2 and transplanted (*i.v.)* with 20×10^6 bone marrow cells and 30×10^6 splenocytes from C57bl/6 (Allo) or Balb /c (Syn) mice. Untreated Balb/c mice were taken as the control group (Cont). (A) The clinical score of aGvHD on day 4 (n=5). (B-C) Serum levels of TNFα (B) and IFNγ (C) were detected using a cytokine array kit at the onset of aGvHD (n=5).

The data are presented as mean ± SEM, *: P < 0.05, **: P < 0.005. (D) Western blot shows the renal levels of NGAL protein in mice with different treatments. Pan 14-3-3 was used as the reference protein.

4.1.2 Accumulation of hetero-lysosomes in the renal proximal tubule epithelial cells

To visualize possible kidney injury, H&E staining was initially conducted. However, no significant histopathological difference was noticed in the kidney between control and aGvHD mice. Then, PAS/PASM staining was applied for its potential to show mesangial matrix, tubular and glomerular basement membrane (137). As shown in Figure 5A, extensive cytoplasmic vacuolization was found in the renal proximal tubule epithelial cells (PTECs) in aGvHD mice compared to those in the control group.

Next, transmission electron microscopy (TEM) was performed to better reveal the pathological changes in the PTECs. In comparison to the control group, tremendous irregular electron-dense heterogeneous contents, suspected as hetero-lysosomes, were seen in the PTECs of mice with aGvHD (Figure 5B). This finding was further validated by staining the tissues with CD68, a protein marker of lysosome. Figure 5C depicts a high expression level of CD68 protein in the PTECs from allo-transplanted mice only, suggesting the abundant accumulation of lysosomes in aGvHD-associated renal injury.

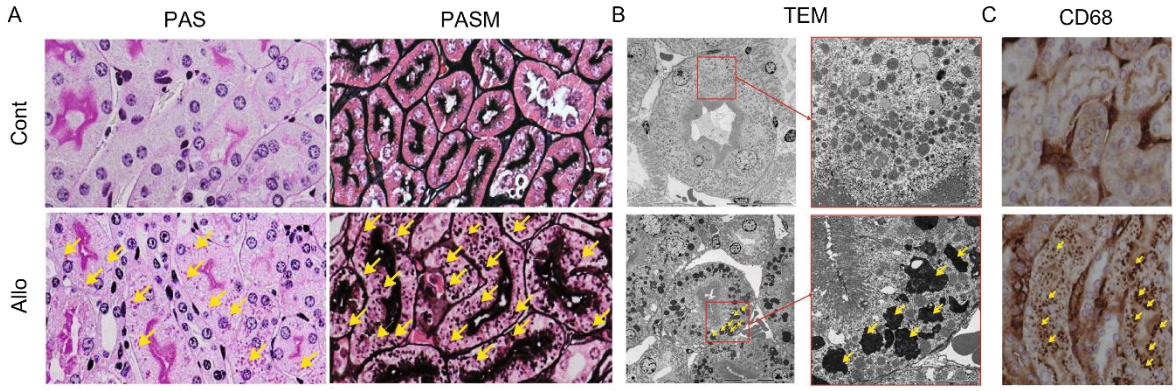

Figure 5. Pathological changes in renal proximal tubule epithelial cells in aGVHD mice

Kidneys from control and aGvHD mice were fixed, embedded, and sectioned. (A) Renal tissues were stained with PAS or PASM. Yellow arrows: argyrophilic vacuolization. (B) Representative kidney sections were imaged using TEM. Yellow arrows: suspected as hetero-lysosomes. (C) Immunohistochemical staining for CD68. Yellow arrows: the site where $CD68^{+}$ granule accumulated. PAS: periodic acid-Schiff base; PASM: periodic acid-Schiff methenamine; TEM: transmission electron microscopy.

4.1.3 Renal αKlotho is downregulated at the onset of aGvHD

αKlotho/FGF23 axis is not only a biomarker for diagnosis and prognosis of kidney disease, but also a pathogenic contributor to the disease development (113). Herein, we studied the alteration of αKlotho and FGF23 in the aGvHD setting to further confirm their roles in kidney injury. As expected, a conspicuous decrease of mRNA encoding αKlotho was found in the kidney of aGvHD mice, while mice in syngeneic group showed a similar expression level as those in the control group (Figure 6A). The corresponding protein was subsequently measured with western blotting, and the same trend was observed (Figure 6B and C). Furthermore, we found significant rises in serum FGF23 in aGvHD mice, which possibly served as a consequence of αKlotho deficiency (Figure 6D). Since αKlotho scarcity has been reported to be associated with increased oxidative stress (117-119), our results indicate that antioxidative strategies might prevent the occurrences of aGvHD and derived kidney injury.

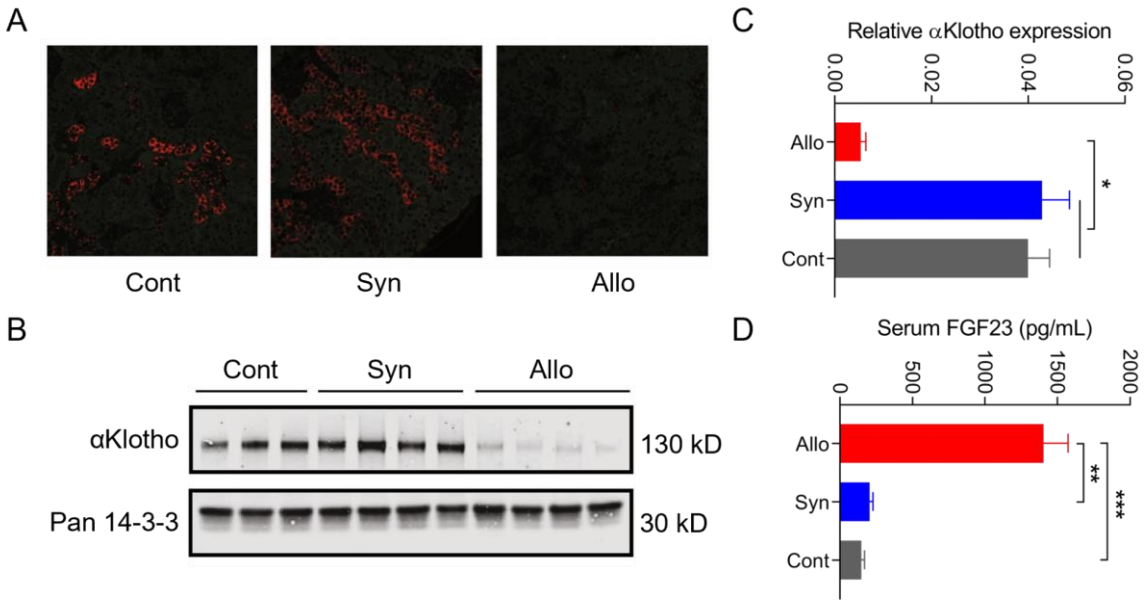

Figure 6. Alteration of αKlotho/FGF23 axis at the onset of aGvHD

(A) Fluorescent *in situ* hybridization of αKlotho in mouse kidney. (B) Protein level of αKlotho in the kidney lysate was detected using western blot. Pan 14-3-3 was applied as the reference protein. (C) Relative protein levels of αKlotho in mouse kidneys. The relative expression is defined as the ratio compared to Pan 14-3-3. (D) Serum levels of FGF23 in mice were determined by ELISA. The data are presented as mean ± SEM (n=5; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$).

4.2 PHARMACOKINETIC STUDY OF NACA

Antioxidative strategies might be utilized for the prophylaxis and treatment of HSCT-related complications like aGvHD as mentioned previously, and cardiovascular side-effects where oxidative stress is also implicated. NACA, the amide derivative of the classic antioxidant NAC, is a promising candidate. However, its pharmacokinetic properties as compared to NAC were

poorly studied. Therefore, we aimed to establish a simple and reproducible analytical method to quantify NACA and NAC in plasma and compare their GSH- replenishing abilities.

4.2.1 NACA and NAC quantification using TCEP/MPOZ derivatization method

Compounds with free thiol residues, like NACA and NAC, are easily oxidized *in vitro* and *in vivo* via forming dimer and conjugating with endogenous thiols like albumin during storage and sample processing. In order to quantify NACA and NAC, TCEP was utilized to cleave disulfide, while the free thiols were stabilized by rapid derivation with MPOZ (Figure 7A). After deproteinization, the extracted samples were measured by LC-MS. The chromatogram in Figure 7B indicates that the current method could identify NACA and NAC simultaneously and selectively.

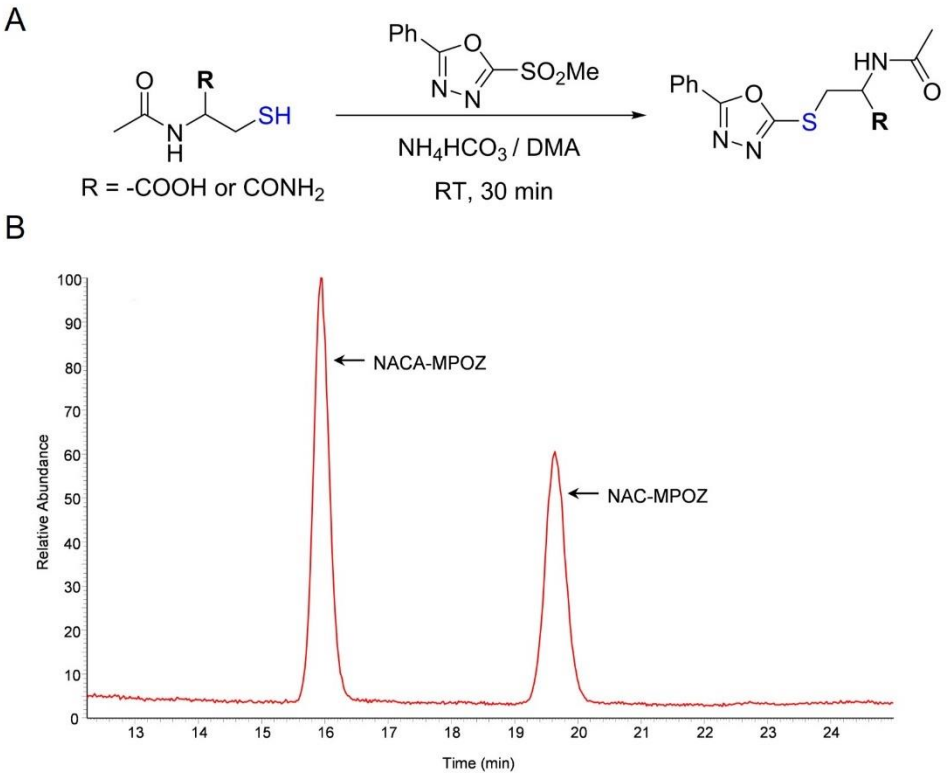

Figure 7. Quantification of NACA and NAC

(A) Reaction scheme for NACA or NAC with MPOZ. (B) LC-MS chromatogram of NACA and NAC in plasma after TCEP/MPOZ derivatization. DMA: dimethylacetamide.

The derivatization method was optimized in terms of reaction time, organic/aqueous (v/v) phase ratios, amounts of TCEP and MPOZ, and the addition sequence of TCEP and MPOZ. Furthermore, the analytical method was validated for specificity, linearity, accuracy, precision, and recovery. Stability assessment indicated that NACA was stable in saline at RT and 4 °C

while less stable in 5% glucose at high concentration at RT, and frequent freeze-thaw procedures should be avoided in NACA-containing plasma sample. Detailed data are available in **Paper II**.

4.2.2 Pharmacokinetic properties of NACA

The aforementioned method was then applied to investigate the pharmacokinetics of NACA. As depicted in Figure 8A, the plasma C_{max} of NACA was 726 μg/mL and 138 μg/mL post intravenous and oral administration at a dose of 300 mg/kg, respectively. In agreement with previous studies (132, 133), our results demonstrated a rapid metabolism of NACA to NAC *in vivo*, as a remarkable conversion to NAC was observed within 10 min after NACA administration (Figure 8B). In addition, we found that the AUCs of the metabolite NAC after intravenous and oral administration of NACA varied, in which the latter route gave a higher (1.7-fold) AUC than the former (Figure 8B).

PK study of NAC was also performed using the same method (curves not shown). Interestingly, the C_{max} of metabolite NAC after oral NACA was 2.5 times higher than that obtained from oral NAC at the same dose (296 μg/mL *vs* 84.0 μg/mL, Figure 8B and Table 3). Such circulating concentration differences could probably predict a superior therapeutical potential of NACA to NAC.

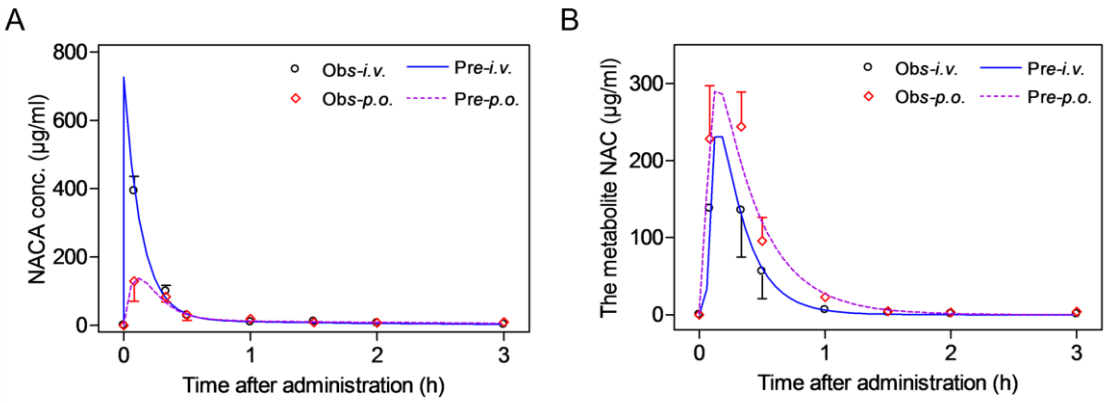

Figure 8. Concentration-time curves of NACA and its metabolite NAC in mouse

NACA was administered intravenously (*i.v.*) and orally (*p.o.*) at a dose of 300 mg/kg. Blood samples were collected at different time points after administration. Concentrations of NACA (A) and its metabolite NAC (B) were measured by LC-MS following TCEP/MPOZ derivatization. Dots: observed (Obs) concentrations after administration, n=3; lines: predicted (Pre) concentrations using WinNonLin software.

The detailed PK parameters of NACA and NAC were calculated and listed in Table 3. We can see that NACA, with the lipophilic character, is eliminated in a biphasic manner in both

administration routes. The distribution phase is rapid with a half-life of around 5 min, but the elimination is relatively longer, with a half-life of 72.8 min for intravenous and 122.7 min for oral administration, respectively. Moreover, NACA showed a higher oral bioavailability compared to NAC (66.8% *vs* 14.6%), which affirms that a lower dose of NACA is needed to achieve the same effect as NAC.

Table 3. Pharmacokinetic parameters for NACA and NAC in mouse

Parameters	NACA		NAC	
	i.v.	*p.o.*	*i.v.*	*p.o.*
AUC (h•μg/mL)	130.4 ± 16.1	87.1 ± 12.4	388.4 ± 38.4	56.8 ± 8.6
V_d (mL)	7.4 ± 1.7	n.a.	3.7 ± 0.5	n.a.
$HL_{1/0}$ (min)	n.a.	n.a.	11.2 ± 1.3	17.8 ± 4.8
α-HL (min)	5.7 ± 0.7	5.0 ± 29.9	n.a.	n.a.
β-HL (min)	72.8 ± 11.1	122.7 ± 29.8	n.a.	n.a.
Cl (mL/h)	41.4 ± 5.1	62.0 ± 8.5	13.9 ± 1.4	98.6 ± 15.0
C_{max} (μg/mL)	726.5 ± 170.2	138.0 ± 42.8	1440.0 ± 190.8	84.0 ± 13.7
Bioavailability (%)	66.8		14.6	

AUC: area under the curve; V_d: volume of distribution; $HL_{1/0}$: elimination half-life in 1-compartment model; α-HL and β-HL: distribution and elimination half-life in 2-compartment model, respectively; Cl: clearance; C_{max}: peak concentration; n.a.: not available.

4.2.3 The GSH-replenishing ability of NACA and NAC

As GSH replenishment is one of the outstanding features of NAC, it is of great interest to investigate such property of NACA in comparison to NAC. Due to the fact that GSH also has a free thiol group, TCEP/MPOZ derivatization together with LC-MS analysis was exploited in GSH measurement as well.

Total GSH increment was defined as the area under relative increment-time curve modeled using WinNonLin. Results showed that the total increment in GSH was 3-4 folds higher after NACA administration compared to that seen from NAC at the same dose (Figure 9). In addition, the level of GSH at 3 h post NAC administration halved that observed from NACA regardless of the administration routes (Figure 9), implying the superior GSH-reinforcing capability of NACA to NAC.

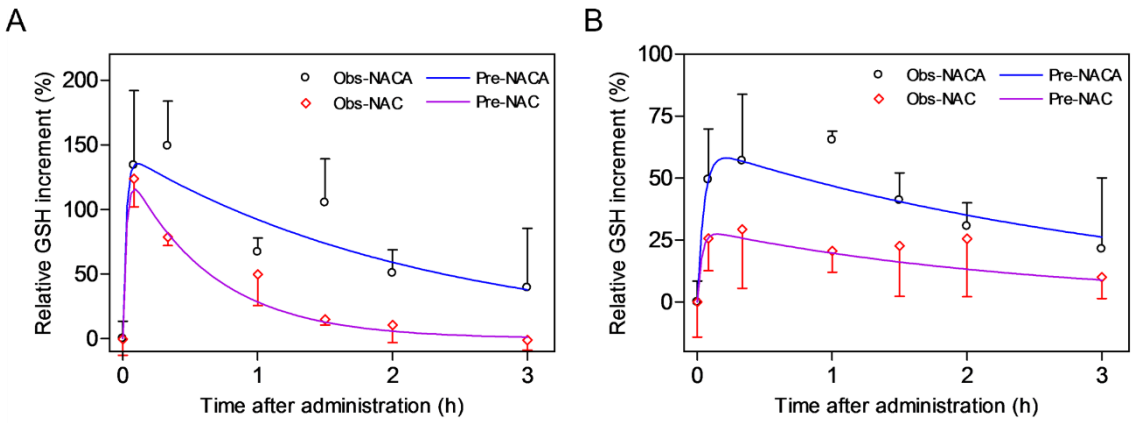

Figure 9. Relative GSH increment after NACA and NAC administration

NACA or NAC was administered intravenously (A) and orally (B) at a dose of 300 mg/kg. Plasma GSH levels were quantified within 3 h post administration. Y-axis displays the percentage of GSH increment compared to the basal level at each time point. Dots: observed (Obs) concentrations after administration, n=3; lines: predicted (Pre) concentrations using WinNonLin software.

4.3 NACA ALLEVIATES THE SEVERITY OF ACUTE GVHD IN MOUSE

Inspired by the superiorities of NACA in oral bioavailability and ability to supplement GSH pool, we investigated its role in mitigating the severity of aGvHD in which oxidative stress is intensively implicated. NAC was compared throughout the study as the reference compound.

4.3.1 NACA reduces the mortality and morbidity of aGvHD

The study relied on a classic aGvHD mouse model as reported previously (96), and NACA treatment was scheduled as listed in Figure 10A. Compared to NAC, intervention with NACA significantly prolonged the survival of mice that underwent allo-HSCT. Specifically, the median survival time in the NACA-treated group was 30 days, whereas it was 22 days in the NAC-treated group which was nearly equal to the control group (Figure 10B). Disease progression was monitored daily using mouse GvHD clinical scoring system (134). As shown in Figure 10C, NACA treatment remarkably reduced the GvHD severity in contrast to the control aGvHD mice, but NAC did not exhibit an obvious protective effect. In addition, histopathological examination showed that mice in the NACA group had a decreased level of aGvHD-target tissue damage, as shown by a thicker hypodermis layer in the skin, less lymphocytes infiltration in the liver, and more regular morphology of colon crypts (Figure 10D).

To evaluate the risk of NACA in interfering with transplantation engraftment, neutrophil and platelet recovery as well as chimerism were examined in recipient mice. Results showed that on day 7 post transplantation, the peripheral neutrophil count was significantly enhanced in the NACA-treated recipients compared to those in NAC and saline groups (Figure 10E). Moreover,

the chimerism was similar among each group, and nearly 100% engraftment was achieved in both bone marrow and spleen on day 14 after BMT (Figure 10F).

Taken together, our results demonstrate that NACA holds the ability to decrease aGvHD severity without hampering hematopoietic reconstitution and engraftment.

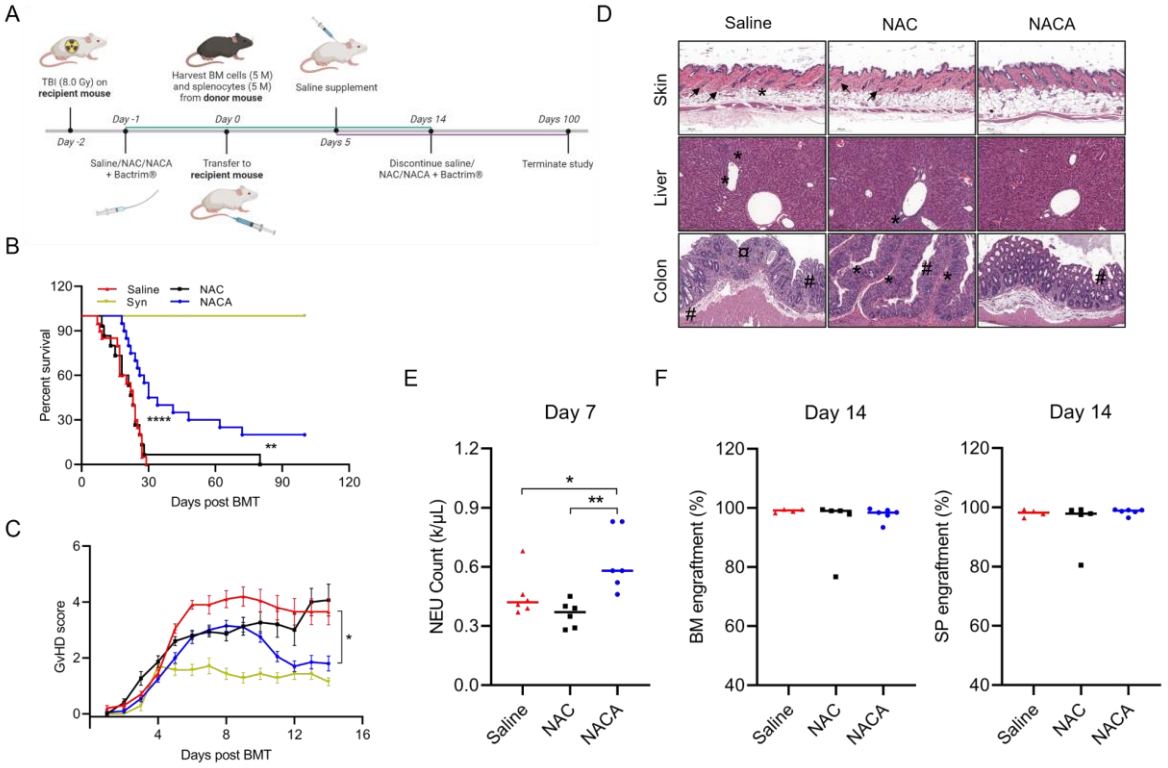

Figure 10. NACA reduces morbidity and mortality of aGvHD

(A) aGvHD mouse model and treatment schedule. (B) Survival of allogeneic/syngeneic-transplanted Balb/c mice (n=15; **: $p < 0.01$; ****: $p < 0.0001$). (C) GvHD clinical scores post HSCT (n=15; *: $p < 0.05$). (D) Representative H&E staining of skin, liver, and colon. Arrow: hair follicle damage or vacuolar changes. *: lymphocytes infiltration. #: apoptosis. ¤: fibrosis with crypts. (E) Neutrophils count on day 7 after HSCT (n=6). (F) Percentages of donor-derived cells on day 14 post HSCT (n=4-6).

4.3.2 NACA confers protection against oxidative stress and inflammation

Next, we investigated the underlying mechanism of NACA in terms of oxidative stress and systemic inflammation which play important roles in the pathogenesis of aGvHD. Firstly, we detected ROS accumulation in splenocytes obtained from recipient mice on day 7 and day 14 after transplantation. Significant downregulation of ROS level was observed in NACA-treated mice in relation to the control aGvHD mice at both time points; however, no change was observed after NAC treatment at the same time points (Figure 11A).

Secondly, serum cytokines were measured in order to assess inflammatory statues of recipient mice. NACA treatment generally lowered the levels of pro-inflammatory cytokines, such as IFNγ, TNFα, IL-1β and IL-6, compared to control aGvHD mice, likewise illustrated in the

levels of IL-2, IL-5, IL-13, IL-17A, IL-17F and IL-22. In contrast, anti-inflammatory cytokine IL-4 was significantly upregulated by NACA administration (Figure 11B). In addition, the frequencies of IFNγ- and TNFα-producing T cells in NACA-treated mice were significantly lower than those in the other two groups on day 7 post transplantation, whereas the difference between NAC and saline groups was not significant (Figure 11C and D).

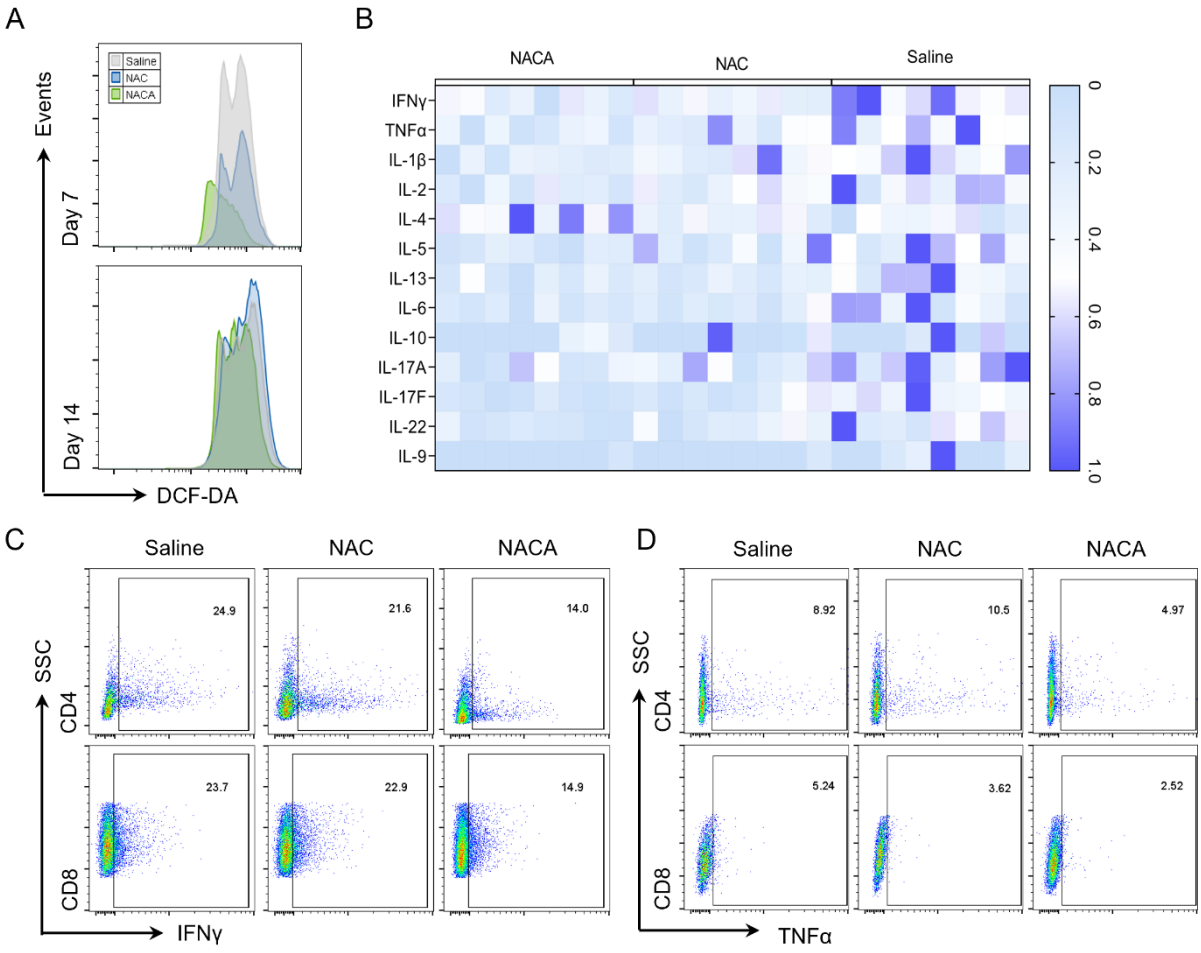

Figure 11. NACA exhibits antioxidative and anti-inflammatory abilities

(A) Splenocytes were harvested on day 7 and 14 after HSCT and analyzed for ROS. (B) Heatmap shows serum cytokine levels in individual mouse from each cohort (n=8). (C and D) Recipient splenic T cells were collected on day 7 and stimulated *in vitro* for 6 h. The expression levels of IFNγ (C) and TNFα (D) in spleen cells were determined.

4.3.3 NACA inhibits the division and migration of graft-derived T-cell

In the context of aGvHD, donor T cells get activated after exposure to allogeneic antigens in secondary lymphoid organs and start to proliferate (138, 139). To investigate the effect of NACA on donor T-cell expansion, recipient mice were transplanted with CellTrace^{TM}-labeled donor splenocytes and euthanized on day 3 for analysis. A modest dividing defect in donor $CD4^+$ and $CD8^+$ T cells was observed in the NACA-treated group compared to the other two groups (Figure 12A). This finding was further validated through staining with the cell

proliferation marker Ki67. We found a significantly higher level of Ki67 expression in mice received saline or NAC than those treated with NACA (Figure 12B).

Host tissue damage is a critical outcome of GvHD and mediated by effector T-cell infiltration (140). In the present study, the levels of chemokine receptor CXCR3 and integrin LPAM-1 (α4β7) were determined for their competences in trafficking T cells from lymphoid tissues and homing T cells to gastrointestinal tract, respectively (141). Compared to the mice treated with saline, those treated with NACA had a decreased expression of CXCR3 and LPAM-1 in both splenic $CD4^+$ and $CD8^+$ cells, indicating a potency of NACA in impeding T cells migration to GvHD-target tissues. However, such phenomenon was not seen upon treatment with NAC (Figure 12C and D). These observations were consistent with our hypothesis and could serve as the testimony for H&E findings in Figure 10D.

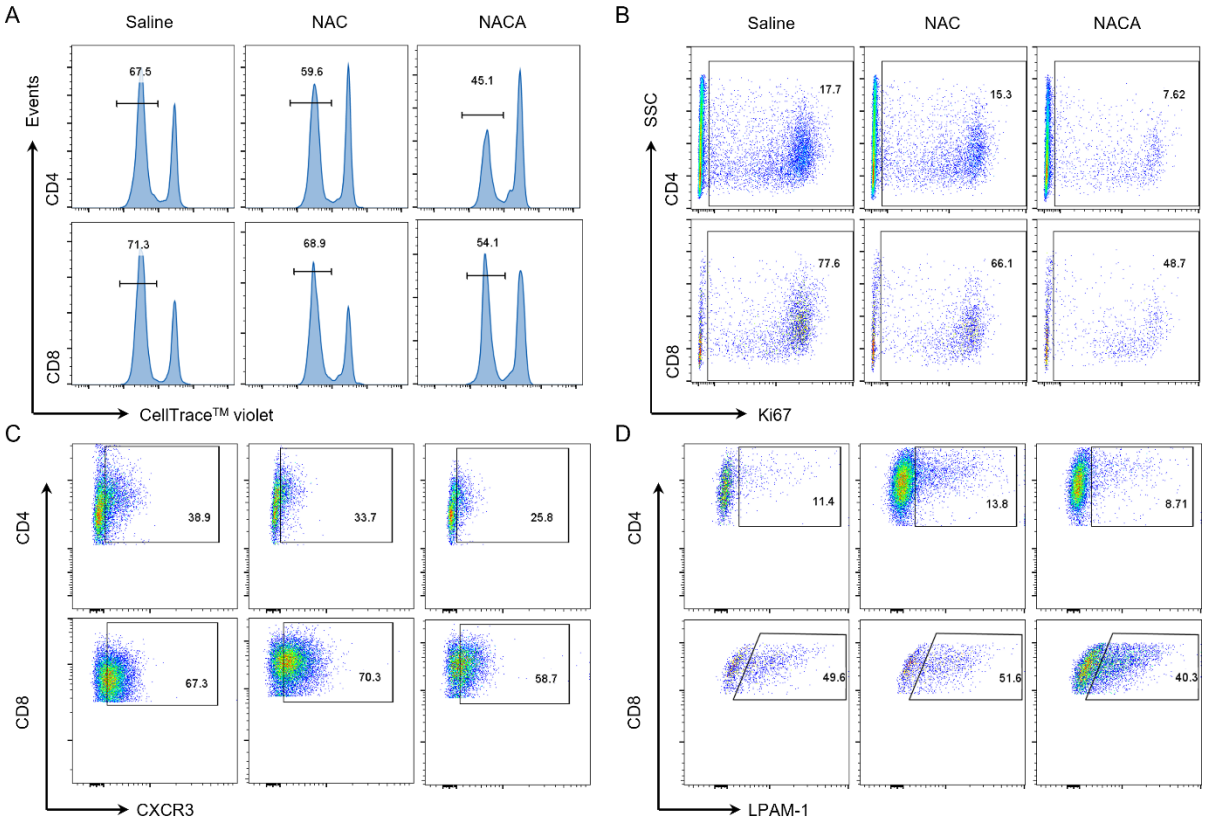

Figure 12. Donor T-cell expansion and migration

(A) Histograms showing donor T-cell division. The gated population refers to divided cells with low levels of CellTrace™ Violet (CellTrace Violet ^{low}). (B) Flow cytometric plots indicate the Ki67 expression in donor T cells. (C-D) Scatter plots show the expression of CXCR3 (C) or LPAM-1 (D) among gated $H2K^{b+}CD4^+$ or $CD8^+$ cells.

4.4 NACA REDUCES CY-INDUCED ENDOTHELIAL DAMAGE

Cardiovascular toxicity is another major complication related to HSCT and accounts for the compromised life quality of transplanted patients, especially the pediatric recipients. As the

inner component of cardiovascular system, endothelium is especially susceptible to damage from radiotherapy and/or cardiotoxic chemotherapy like Cy. Since elevated oxidative stress was found to be one of the major causes and associated with other mechanisms underlying Cy-induced cardiotoxicity, it is promising to explore the ability of NACA to attenuate endothelial injury caused by Cy.

4.4.1 *In vivo* protective effect of NACA

Balb/c mice received a single dose of Cy on day 0, and the prophylactic treatment with NAC or NACA was given from day -7 to day 7, but not day 0 (Figure 13A). H&E staining was performed on aorta sections to visualize possible damage in the endothelium. Clear endothelial nuclei were shown on the inner layer of the aorta of control mice without treatment, and the tunica media was supported by arranged smooth muscle cells and linked to tight junctions with tunica intima. Administration of 400 mg/kg Cy caused conspicuous detachment of the endothelial nuclei along with an irregular arrangement of the matrix fiber and smooth muscle cells in the middle elastic lamina. Both NACA and NAC treatments showed the ability to restrain the impairment, although with different degrees (Figure 13B).

We further investigated the expression level of ICAM-1 which marks endothelial injury and inflammatory responses (142). The results reveal that prophylactic treatment with NACA decreased the aortic endothelial ICAM-1 protein levels that were upregulated by high-dose Cy. NAC-treated mice exhibited less decreases in the level of ICAM-1expression (Figure 13C).

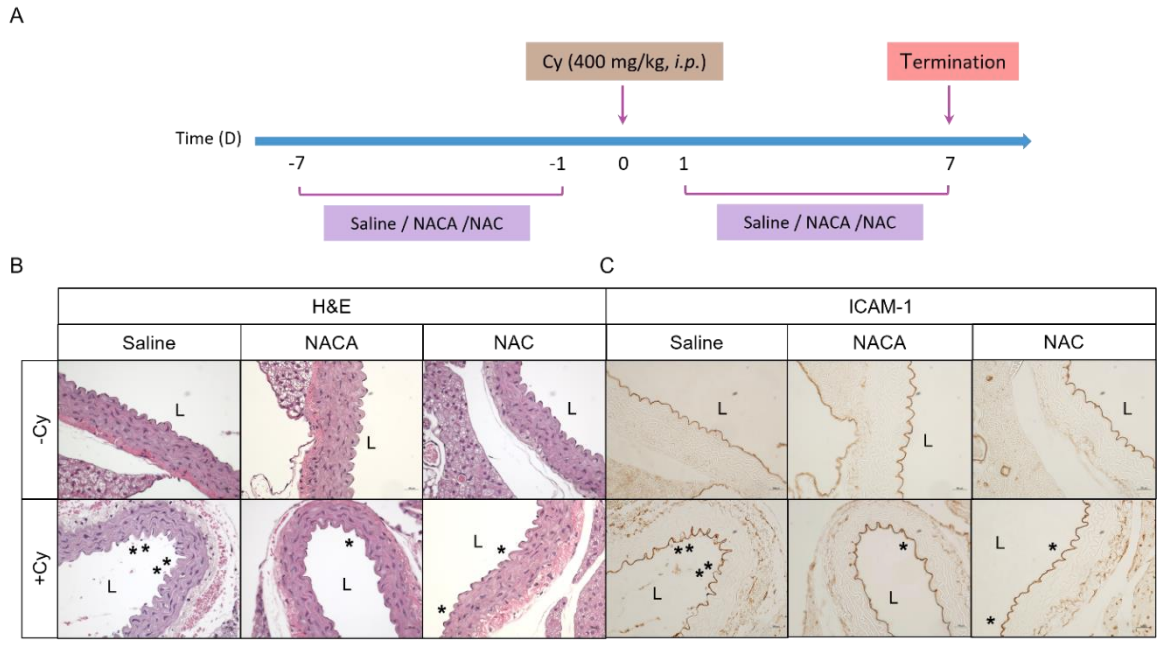

Figure 13. NACA mitigates endothelial damage caused by Cy

(A) Treatment schedule. (B) Representative H&E staining on aortas. L: lumen; *: suspected damaged site. (C) Representative immunohistochemical staining with ICAM-1 antibody. *: ICAM-1 protein high-expressing site.

4.4.2 Endothelial cell viability and DNA damage

To better understand how Cy impairs vascular endothelium and NACA's mechanism of action, *in vitro* studies were performed on human endothelial cells. The prodrug Cy is activated by cytochrome P450 enzymes to cytotoxic metabolite, 4-hydroxycyclophosphamide (4-OH-Cy), while human endothelial cells lack major cytochrome P450 enzymes. Therefore, MAF was used to produce 4-OH-Cy through automatic hydrolysis in aqueous phase.

Crystal violet staining was conducted to quantify cell mass. Both NACA and NAC protected endothelial cells against 4-OH-Cy-induced cell death, yet NACA was superior to NAC at the same concentration. Strikingly, 5 mM of NACA pre-treatment nearly protected all HAECs upon exposure to 4-OH-Cy (Figure 14A and B). As one of the classic pathways of cell death, apoptosis was evaluated in different conditions. It was shown that 1 mM of NACA significantly decreased the apoptosis of both cell lines, whereas there was no effect for 1 mM of NAC. When the concentration was raised to 5 mM, both antioxidants demonstrated significant protective capability on endothelial cells, but NAC was still inferior to NACA (Figure 14C and D).

Cy acts partially through causing irreversible DNA double strands break, thus, the effects of NACA and NAC on 4-OH-Cy-induced DNA damage were compared. Results show that both antioxidants reduced DNA damage in a dose-dependent manner, and NACA showed a greater capability than NAC. It is noteworthy that the DNA damage caused by 4-OH-Cy was almost fully abrogated by 5 mM of NACA in both cell lines (Figure 14E and F).

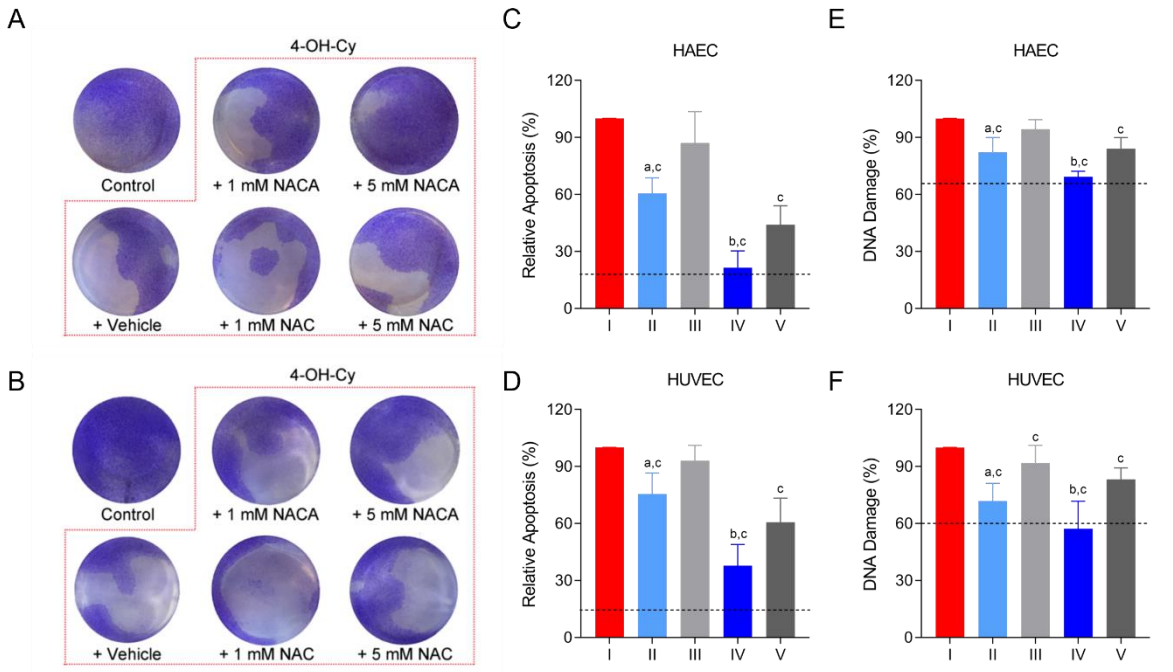

Figure 14. NACA attenuates 4-OH-Cy-induced endothelial cell death and DNA damage

Endothelial cells were pre-treated with NACA or NAC for 6 h and were further treated with 4-OH-Cy for 20 h. HAECs (A) and HUVECs (B) were fixed and subjected to crystal violet staining. (C-D) Relative apoptotic cells upon each treatment. Cells were stained by PE-Annexin V apoptosis detection kit I, and the apoptosis rate was defined as the sum percentage of early (Annexin $V^+7\text{-}ADD^-$) and late (Annexin

$V^+7\text{-}ADD^+$) apoptotic cells. The apoptosis rate in the group treated with 4-OH-Cy was set to 100% (n=5). (E-F) Relative DNA damage in endothelial cells. The percentage of DNA damage in the group treated with 4-OH-Cy was considered as 100% (n=5). Dash lines refer to data in control group without treatment. I: 4-OH-Cy; II: 1 mM NACA + 4-OH-Cy; III: 1 mM NAC + 4-OH-Cy; IV: 5 mM NACA + 4-OH-Cy; V: 5 mM NAC + 4-OH-Cy. Results are presented as the mean ± SD. a: $p < 0.05$ when it was compared to III; b: $p < 0.05$ when it was compared to V; c: $p < 0.05$ when it was compared to I.

4.4.3 NACA decreases 4-OH-Cy-induced oxidative stress

To elucidate the role of NACA in 4-OH-Cy-induced oxidative stress, cellular ROS production and intracellular antioxidative defense system were investigated. As depicted in Figure 15A and B, excessive ROS was found in HAECs and HUVECs post 4-OH-Cy treatment using DCD-DA probe. In line with our previous results, pre-incubation with 1 mM of NACA significantly reduced ROS accumulation in 4-OH-Cy-treated cells; however, NAC worked only at a higher concentration.

In addition, we found that in response to 4-OH-Cy treatment, the activities of two enzymatic antioxidants (i.e., catalase and SOD) and the content of reduced GSH were compromised to a large extent, especially in HAECs. Both 1 mM and 5 mM of NACA remarkably enhanced the enzyme activities and reinforced GSH level in endothelial cells, and the higher concentration of NACA almost restored catalase and SOD to normal levels. In comparison, 1 mM of NAC was not able to cope with the unfavorable alteration, and a concentration of 5 mM only partially strengthened the antioxidative defense system (Figure 15C-F).

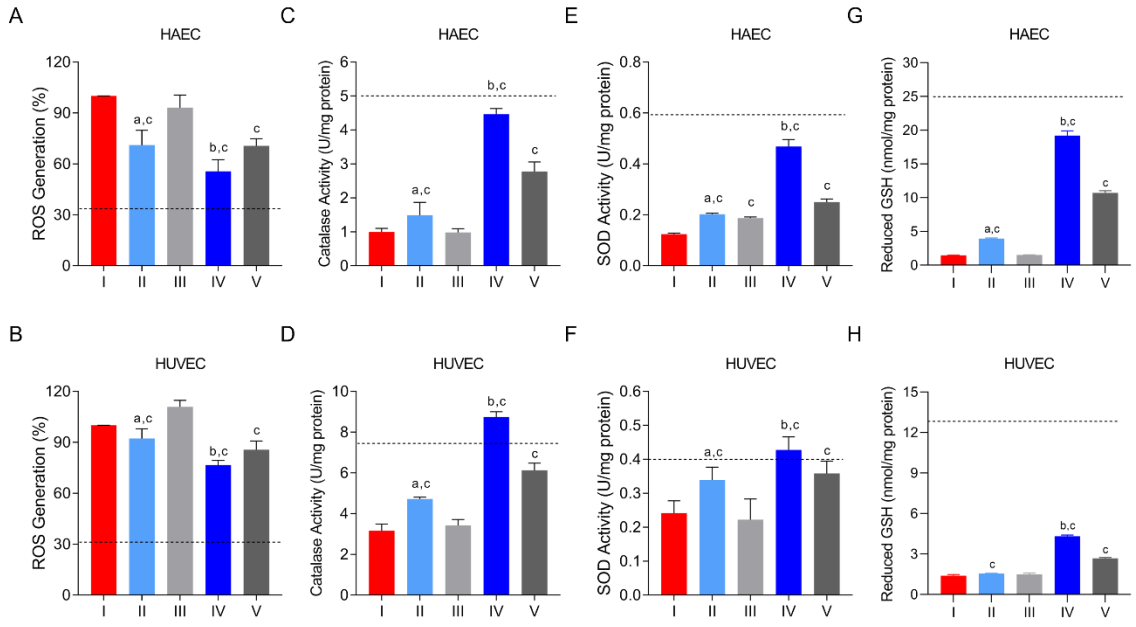

Figure 15. NACA protects endothelial cells against 4-OH-Cy-induced oxidative stress

ROS levels in HAECs (A) and HUVECs (B) after 4-OH-Cy exposure with or without antioxidants pre-incubation. The amount of ROS generation in the group treated with 4-OH-Cy was set to 100% (n=5). (C-D) Absolute catalase activity in endothelial cells (n=5). (E-F) Absolute SOD activity in endothelial cells (n=5). (G-H) The content of intracellular reduced GSH (n=4). Dash lines refer to the level in control

group without treatment. I: 4-OH-Cy; II: 1 mM NACA + 4-OH-Cy; III: 1 mM NAC + 4-OH-Cy; IV: 5 mM NACA + 4-OH-Cy; V: 5 mM NAC + 4-OH-Cy. Results are presented as the mean ± SD. a: $p < 0.05$ when it was compared to III; b: $p < 0.05$ when it was compared to V; c: $p < 0.05$ when it was compared to I.

4.4.4 NACA abrogates eNOS/arginase imbalance and preserves angiogenesis

eNOS and arginases are critical for NO synthesis. Since NO plays an important role in maintaining vascular tone and endothelial hemostasis, the levels of eNOS and arginases in endothelial cells were assessed. We found that compared to the control group, 4-OH-Cy treatment significantly reduced eNOS protein expression and increased the level of arginase I in HAECs but increased the expression levels of both proteins in HUVECs. These changes were reversed in cells treated with 5 mM of NACA, while the effects of NAC were not significant. Interestingly, neither NACA nor NAC pre-treatment improved the protein level of arginase II that was downregulated by 4-OH-Cy (Figure 16A).

Next, a tube formation assay was performed as an *in vitro* investigation of angiogenesis. Our results reveal that capillary tubular structures formed by both cell lines on BME matrix were significantly inhibited by 4-OH-Cy, resulting in a shorter total tube length, a smaller area of meshes, and a greater number of extremities. NACA (5 mM) improved the tube formation capability to a level comparable to that observed in untreated cells (Figure 16B-C).

To further explore the underlying mechanism of NACA in 4-OH-Cy-induced angiogenesis dysfunction, the Notch signaling was investigated as it has been proven to be a pivotal factor for sprouting angiogenesis and vessel maturation (143). It is depicted that the expression levels of Notch-1, Jagged-1, and their downstream product HES-1 in HAECs and HUVECs were significantly decreased following exposure to 4-OH-Cy, and pre-treatment with 5 mM of NACA or NAC partially reversed these effects (Figure 16D).

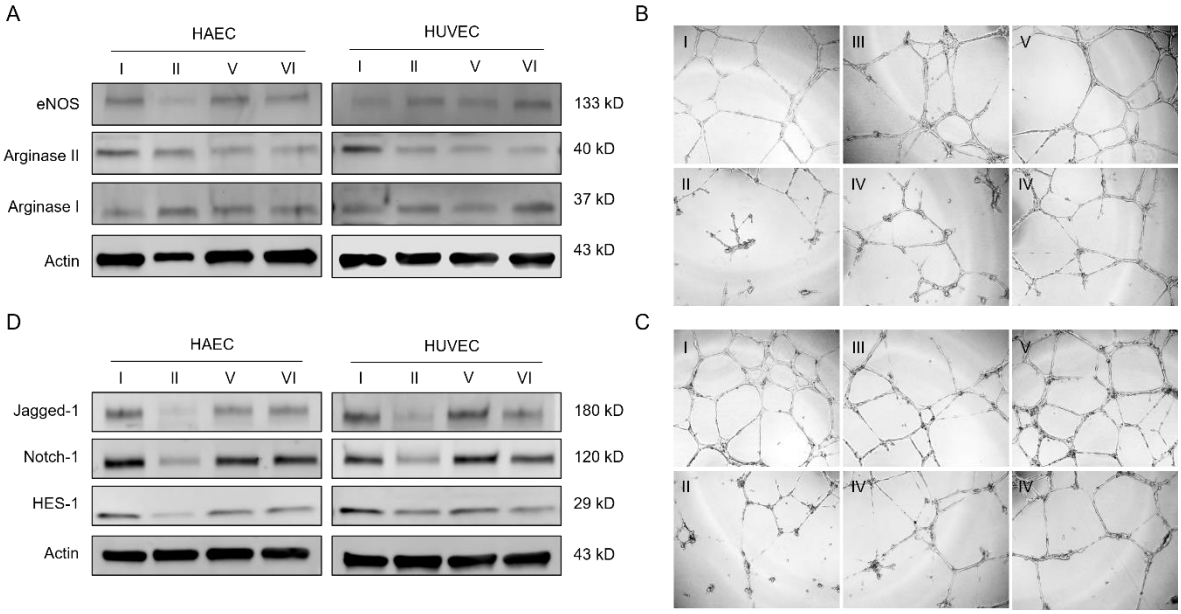

Figure 16. Western blot analysis and *in vitro* angiogenesis assay

(A) Western blot analysis of eNOS and arginases in endothelial cells. (B-C) Representative phase-contrast micrographs of tubular structures in cultured HAECs (B) and HUVECs (C) after different treatments. (D) Western blot analysis for the expression of Jagged-1, Notch-1, and HES-1. I: control; II: 4-OH-Cy; III: 1 mM NACA + 4-OH-Cy; IV: 1 mM NAC + 4-OH-Cy; V: 5 mM NACA + 4-OH-Cy; VI: 5 mM NAC + 4-OH-Cy.

5 DISCUSSION

5.1 KLOTHO, A NEW TARGET FOR ACUTE GVHD

There are tremendous evidence showing that AKI is a common adverse effect following allogeneic HSCT, and the incidence is as high as 55.1% according to a meta-analysis (144). Multiple factors including infection, sinusoidal obstructive syndrome, thrombotic microangiopathy and the use of nephrotoxic drugs have been ascribed as the contributors to AKI development in transplanted patients (145). aGvHD is a potential risk factor in AKI, but this notion has not been clearly elucidated. Using a major histocompatibility complex (MHC) mismatched GvHD mouse model, we confirmed the association between renal injury and the onset of aGvHD.

Our first proof was the increased renal NGAL expression in mice with aGvHD when compared to those underwent syngeneic transplantation (Figure 4D). NGAL, a protein initially discovered in the activated neutrophils, is a biomarker of kidney damage (146). Physiologically, NGAL promotes epithelial differentiation and leads to glomeruli and proximal/distal tubules generation (147). However, elevated NGAL production and release is usually observed after inflammatory stimuli (148). Therefore, amplified levels of NGAL in kidney suggest that the onset of aGvHD probably is an underlying cause of AKI and kidney is a potential target organ for aGvHD. This hypothesis was validated by histopathological examination (Figure 5A), in which numerous cytoplasmic vacuolization was found in the renal PTECs of aGvHD mice.

Another piece of evidence was the accumulation of hetero-lysosomes in the renal PTECs observable via TEM and CD68 staining (Figure 5B and C). Lysosome is responsible for degradation and recycling of intracellular and extracellular macromolecules (149). Once broken down, the lysosomal catabolites need to be transported out and make room for new components (150). Accumulation of hetero-lysosomes in the proximal tubules implies a lysosome dysfunction in the kidney. This can be partially reflected by the increased level of full-length NGAL (Figure 4D) which is normally degraded by renal lysosome (151).

The most valuable finding in **Study I** was the significant reduction of αKlotho and escalation of FGF23 in kidneys of aGvHD mice (Figure 6). Given that αKlotho downregulation is constantly implied in AKI and CKD (113, 135), our results further verified the kidney injury in the context of aGvHD. Moreover, several preclinical studies counteracting αKlotho deficiency in kidney disorder and extrarenal complications have shown promising results (152-155). The existing evidence together with our findings motivates us to consider αKlotho as a potential target for aGvHD prophylaxis and therapy. However, the safety profile and efficacy of the proposed methods like gene editing, epigenetic modulation and Klotho replacement have not been well established in humans (155). Besides the connection to inflammation, αKlotho deficiency is also associated with oxidative stress. Thus, it is likely that strategies aiming to improve the antioxidative defense system could be a promising alternative to prevent aGvHD.

5.2 PHARMACOKINETIC ADVANTAGE OF NACA

Off-label use of approved drugs to pharmacologically restore redox homeostasis remains a feasible method. Among the clinically used redox active substances, NAC is particular of our interest due to its intensive application as a classic antioxidant. We hypothesize that the amide derivative NACA is more promising because of its higher lipophilicity and cell membrane-permeability. To prove our hypothesis and understand the advantages of NACA over NAC, it is of great urgency to compare the PK characteristics of both compounds comprehensively.

Since NAC is reported to be a metabolite of NACA, a quantitative method that can adequately measure both simultaneously was warranted. Although two analytical methods have been reported for this purpose (132, 133), the accuracy was not satisfactory. Herein, we developed a novel method featuring TCEP/MPOZ derivation and LC-MS detection to enable quantification of NACA and NAC in a rapid and precise manner (Figure 7).

Using this newly established method, we depicted the PK behavior of NACA for the first time. In line with previous studies (132, 133), our data demonstrate an instant metabolism of NACA to NAC after systemic administration. Interestingly, we found that the AUC of the metabolite NAC was higher when NACA was given orally in relation to that obtained from an equal intravenous dose (Figure 8B). This finding clearly shows first-pass effect, suggesting that NACA is predominately metabolized in liver. Additionally, the PK behavior of NACA followed a two-compartment open model with fast distribution and relatively slow elimination (Table 3). This further implies that NACA rapidly distributes to different tissues, most probably benefiting from its superior lipophilicity. Our findings can, at least partially, explain the antioxidative efficacy of NACA in various organs (131, 156).

When comparing the PK properties of NACA with NAC, we discovered that NACA holds a superior bioavailability to NAC (66.8% *vs* 14.6%). This is of great clinical importance, because it means a lower dose of NACA could reach the same therapeutic index compared to NAC. Interestingly, regardless of the administration route, NACA was found to replenish GSH, the major reducing equivalent, at a higher efficiency over NAC. Overall, our data attests that NACA is more favorable than NAC in PK behaviors, predicting an improved antioxidative effect.

5.3 APPLICATION IN ACUTE GVHD

Our study reveals that NACA, rather than NAC, has the ability to protect mice from aGvHD post allo-HSCT to some extent. Nevertheless, due to the fact that NACA is converted to NAC after systemic administration, plausible reasons need to be given to explain the observed discrepancies.

Firstly, NACA possessed a better antioxidative potency than NAC. As shown in our study, NACA continuously decreased ROS accumulation in the spleen of aGvHD mice, whereas

NAC did not exhibit a similar effect (Figure 11A). This is perhaps because of the structural and physicochemical differences between NAC and NACA. The hydrophilic feature of NAC renders it more difficult to cross splenocytes membrane, resulting in inadequate NAC to scavenge massive ROS during the process of aGvHD. In contrast, the hydrophobicity of NACA endows higher cell membrane-permeability as well as antioxidative capacity. Likewise, a higher level of circulating GSH was attained by NACA compared to NAC at the same dose (Figure 9). Since GSH is either a direct electrophilic and oxidant species scavenger or act through enzymatic catalysis (157), our results imply NACA as a more powerful antioxidant.

Secondly, NACA demonstrated a stronger anti-inflammatory capacity compared to NAC, as evident by lower levels of pro-inflammatory cytokines (e.g., TNFα, IFNγ and IL-17A) and elevated level of anti-inflammatory cytokine (i.e., IL-4) (Figure 11B). The anti-inflammatory effect of NACA may not be expounded independently from its antioxidative nature, because oxidative stress can activate transcription factors and pro-inflammatory genes and arouse inflammation (158). Moreover, oxidative condition can induce the generation of oxygen-specific epitopes that act as damage-associated molecular patterns and trigger inflammation (159). Accordingly, better antioxidative ability of NACA can largely reflect superior anti-inflammatory performance.

Thirdly, since NAC has been reported to have immunostimulatory capacity at low dose while with immunosuppressive competence at a high dose (126), we speculate that with higher oral bioavailability, the same dose of NACA is equivalent to a higher dose of NAC, thus immunosuppression should dominate after NACA administration. This speculation was testified by restrained donor T-cell division and proliferation, polarization, and migration (Figure 12, and figures in **Paper III**). The obtained results have also provided further evidence to show the immunomodulatory ability of NACA.

Collectively, the diverse arsenal of mechanisms may provide NACA an advantage over the conventionally used NAC, especially against a disease like aGvHD which itself is underpinned by a complex pathology. Albeit NACA showed a moderate effect on aGvHD when used alone, it would be more beneficial to incorporate NACA into the conventional aGvHD prophylactic strategies.

5.4 CARDIOVASCULAR IMPLICATIONS

High dose of Cy has been reported to be associated with cardiovascular toxicity. In the *in vivo* section of **Study IV**, we found that NACA maintained the integrity of the aorta endothelium and downregulated the expression of ICAM-1 following Cy exposure (Figure 13). Intact vascular endothelium is a gatekeeper of cardiovascular health, whereas endothelial disturbance could contribute to diverse cardiovascular ailments, such as atherosclerosis, hypertension, and diabetes (160). Therefore, abrogation of endothelial dysfunction may impede the development of cardiovascular disorders and is of great clinical relevance. Moreover, since ICAM-1 is one

of the major adhesion molecules mediating leukocytes recruitment from blood to inflammatory sites, decreased levels of ICAM-1 is linked to less endothelial cell leakage and activation (161), suggesting a decreased inflammation after NACA treatment.

The *in vitro* studies showed that NACA protected endothelial cells from 4-OH-Cy-induced death. This effect is achieved through not only alleviating DNA damage but also strengthening the antioxidative defense system (Figure 14 and 15). Similar to the phenomenon observed in the aGvHD mouse model, pre-treatment with NACA partially neutralized intracellular ROS accumulated in experimental endothelial cells. Moreover, NACA partly restored the GSH pool. Besides the antioxidative feature, GSH also participates in the detoxication of cytostatic compounds by covalently modification (162). The powerful GSH-reinforcing ability could facilitate NACA to mitigate the toxicity caused by 4-OH-Cy.

In addition, NACA rebalanced eNOS/arginase hemostasis that was disrupted by 4-OH-Cy treatment (Figure 16A). On one hand, a critical function of endothelium is to keep the vascular tone that largely depends on NO bioavailability. NO synthesis is accomplished by eNOS along with the co-factor tetrahydrobiopterin (BH4) using L-arginine as the substrate. But L-arginine is also consumed by arginases; thus, rebalanced eNOS and arginase by NACA is essential to ensure NO production. On the other hand, insufficient L-arginine or BH4 will result in uncoupled eNOS, causing massive ROS production (163). Since redundant ROS will in turn exacerbate eNOS/arginase imbalance (164), the more formidable antioxidative ability confers NACA a better efficacy to inhibit the formation of uncoupled eNOS.

Using a tube formation assay, we found that the angiogenic property of endothelial cells was wrecked by 4-OH-Cy treatment, and inhibition of Notch signaling pathway was at least one of the underlying mechanisms (Figure 16B-D). Since suppressed Notch signaling is associated with oxidative stress and vice versa (165), strategies focusing on scavenging ROS might display a protective effect against 4-OH-Cy-induced angiogenesis deficiency. Indeed, the disturbance was partially prevented by 5 mM of NAC but was fully abrogated by 5 mM of NACA.

6 CONCLUSIONS

In the present thesis, we explored a new prophylactic strategy to ameliorate HSCT-related complications. Firstly, we confirmed that the renal injury occurs at the onset of aGvHD, with the features of NGAL upregulation, hetero-lysosomes accumulation in the renal PTECs, abrogated αKlotho and increased level of circulatory FGF23. These findings suggest that approaches aiming to restore αKlotho content, or with anti-inflammatory and antioxidative effects might be able to prevent aGvHD.

NACA, a small-molecule drug with powerful antioxidative ability, was selected for further investigation. We developed a robust, selective, and reproducible analytical method to quantify NACA and its downstream products NAC and GSH in order to fully elucidate its therapeutic potency. We found that NACA was rapidly metabolized to NAC *in vivo*, and its kinetics fitted to a two-compartment open model. Importantly, NACA was superior to NAC regarding bioavailability and GSH-reinforcing ability.

In pursuit of the prophylactic efficacy of NACA in aGvHD setting, a classic MHC major mismatched transplantation mouse model was applied. We found that NACA significantly reduced the mortality and morbidity in aGvHD mice without altering the engraftment. In addition, this promise was mediated by multifunctional mechanisms including antioxidation, anti-inflammation, and immunomodulation. Moreover, NACA displayed better prophylactic effects than NAC.

In parallel, NACA showed a protective ability in chemotherapy-induced endothelial dysfunction. It preserved the integrity of vascular endothelium in Cy-treated mice and downregulated local ICAM-1expression. NACA restrained apoptosis, DNA damage, and oxidative stress, and restored eNOS and arginase I in endothelial cells. Furthermore, the neovascularization of endothelial cells was salvaged by NACA through modulating Notch signaling pathway. Although NAC was effective in some cases, the overall therapeutic efficacy was not comparable to that obtained from NACA.

Together, this thesis reveals that antioxidant treatment, such as NACA, is a promising prophylactic strategy in management of HSCT-related complications. Our findings emphasize the possibility of redox hemostasis as a therapeutic target for such pathological conditions.

7 POINTS OF PERSPECTIVE

In **Study I**, the phenotype that significant αKlotho deficiency was observed at the onset of aGvHD. However, experiments with longer follow-up should also be performed to determine the long-term effect on the kidney. In addition, further investigations are required to evaluate whether αKlotho replacement strategies, like administration of recombined αKlotho, are effective in reducing the severity of aGvHD-derived renal injury. Furthermore, as hetero-lysosomes were found to accumulate in PTECs at the onset of aGvHD, investigations with the aim to explore the underlying mechanism are warranted.

As seen in **Study II**, NACA is rapidly eliminated from circulation after administration. It has been reported that formulating NACA as a co-crystal with an excipient may endow a longer half-life in plasma (166). In order to enhance the pharmacokinetic and pharmacodynamic behavior of NACA, the development of different pharmaceutical formulations might be helpful.

Study III showed a moderate prophylactic effect of NACA against aGvHD in mouse model. Our results are still preliminary, and several questions need to be answered before the translation from bench to bedside. Firstly, the mechanism through which NACA reduced oxidative stress in aGvHD has not been fully understood, and relevant *in vitro* studies would be beneficial. Secondly, our results should be validated in other GvHD mouse models, such as MHC minor mismatched or matched model. Thirdly, the effect of NACA on graft-versus-leukemia, a beneficial immune reaction to eradicate the primary malignancy and prevent tumor relapse, remains unexplored. Moreover, with a moderate effectiveness, NACA should not be suggested to be used alone in aGvHD. A better option is to include NACA as an adjuvant treatment on top of clinically used strategies, like PTCy and calcineurin inhibitor, to increase their efficacy and decrease their dose-related toxicity. Therefore, future endeavors should be devoted to exploring the feasibility of combination treatment.

Although **Study IV** elucidated the protective effect of NACA in Cy-induced endothelial injury, endothelial function has not been fully assessed. To overcome this limitation, *ex vivo* myography method could provide more straightforward and convincing evidence. As smooth muscle cells/cardiomyocytes are another major component of the cardiovascular system, it is equally compulsory to examine the effect of NACA in those cells. Moreover, imaging modalities like echocardiography and cardiac magnetic resonance imaging will help better recognize the general cardiac and endothelial health *in vivo*. Last but not least, further adequately powered prospective studies are required to confirm whether the prophylactic NACA treatment will interfere with the therapeutic aim of Cy.

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9 REFERENCES

1. Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, et al. Cancer statistics for the year 2020: An overview. International journal of cancer. 2021.

2. Cancer registration & surveillance modules U. S. : National Institutes of Health, National Cancer Institute; [Available from: <https://training.seer.cancer.gov/>.

3. Thomas ED. A history of haemopoietic cell transplantation. British Journal of Haematology. 1999;105(2):330-9.

4. Sahdev I, Abdel-Azim H. Chapter 31 - Hematopoietic Stem Cell Transplantation. In: Lanzkowsky P, Lipton JM, Fish JD, editors. Lanzkowsky's Manual of Pediatric Hematology and Oncology (Sixth Edition). San Diego: Academic Press; 2016. p. 577-604.

5. Niederwieser D, Baldomero H, Bazuaye N, Bupp C, Chaudhri N, Corbacioglu S, et al. One and a half million hematopoietic stem cell transplants: continuous and differential improvement in worldwide access with the use of non-identical family donors. Haematologica. 2022;107(5):1045-53.

6. Passweg JR, Baldomero H, Chabannon C, Corbacioglu S, de la Cámara R, Dolstra H, et al. Impact of the SARS-CoV-2 pandemic on hematopoietic cell transplantation and cellular therapies in Europe 2020: a report from the EBMT activity survey. Bone Marrow Transplantation. 2022;57(5):742-52.

7. Auletta J.J. KJ, Chen M., Shaw B.E. Current use and outcome of hematopoietic stem cell transplantation: CIBMTR US summary slides. 2021.

8. Gyurkocza B, Sandmaier BM. Conditioning regimens for hematopoietic cell transplantation: one size does not fit all. Blood. 2014;124(3):344-53.

9. Hatzimichael E, Tuthill M. Hematopoietic stem cell transplantation. Stem cells and cloning : advances and applications. 2010;3:105-17.

10. Bhattacharya N, Mukherijee K, Chettri MK, Banerjee T, Mani U, Bhattacharya S. A study report of 174 units of placental umbilical cord whole blood transfusion in 62 patients as a rich source of fetal hemoglobin supply in different indications of blood transfusion. Clinical and experimental obstetrics & gynecology. 2001;28(1):47-52.

11. Keating AK, Langenhorst J, Wagner JE, Page KM, Veys P, Wynn RF, et al. The influence of stem cell source on transplant outcomes for pediatric patients with acute myeloid leukemia. Blood advances. 2019;3(7):1118-28.

12. Faiz SA, Grosu HB, Shannon VR. Pulmonary Complications of Cancer Therapy. In: Kantarjian HM, Wolff RA, editors. The MD Anderson Manual of Medical Oncology, 3e. New York, NY: McGraw-Hill Medical; 2016.

13. Linschoten M, Kamphuis JAM, van Rhenen A, Bosman LP, Cramer MJ, Doevendans PA, et al. Cardiovascular adverse events in patients with non-Hodgkin lymphoma treated with first-line cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) or CHOP with rituximab (R-CHOP): a systematic review and meta-analysis. The Lancet Haematology. 2020;7(4):e295-e308.

14. Ishiguro H, Masuda N, Sato N, Higaki K, Morimoto T, Yanagita Y, et al. A randomized study comparing docetaxel/cyclophosphamide (TC), 5-fluorouracil/epirubicin/cyclophosphamide (FEC) followed by TC, and TC followed by FEC

for patients with hormone receptor-positive HER2-negative primary breast cancer. Breast Cancer Research and Treatment. 2020;180(3):715-24.

15. Ogino MH TP. Cyclophosphamide: Treasure Island (FL): StatPearls Publishing; 2022.

16. Gyurkocza B, Sandmaier BM. Conditioning regimens for hematopoietic cell transplantation: one size does not fit all. Blood. 2014;124(3):344-53.

17. Mielcarek M, Furlong T, O'Donnell PV, Storer BE, McCune JS, Storb R, et al. Posttransplantation cyclophosphamide for prevention of graft-versus-host disease after HLA-matched mobilized blood cell transplantation. Blood. 2016;127(11):1502-8.

18. Leimi L, Jahnukainen K, Olkinuora H, Meri S, Vettenranta K. Early vascular toxicity after pediatric allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplantation. 2022;57(5):705-11.

19. Wallhult E QB. The European Blood and Marrow Transplantation Textbook for Nurses: Under the Auspices of EBMT.: Cham (CH): Springer; 2018.

20. Majhail NS. Long-term complications after hematopoietic cell transplantation. Hematol Oncol Stem Cell Ther. 2017;10(4):220-7.

21. Cappelli B, Volt F, Tozatto-Maio K, Scigliuolo GM, Ferster A, Dupont S, et al. Risk factors and outcomes according to age at transplantation with an HLA-identical sibling for sickle cell disease. Haematologica. 2019;104(12):e543-e6.

22. Hierlmeier S, Eyrich M, Wölfl M, Schlegel P-G, Wiegering V. Early and late complications following hematopoietic stem cell transplantation in pediatric patients – A retrospective analysis over 11 years. PLOS ONE. 2018;13(10):e0204914.

23. D'Souza A FC. Current Uses and Outcomes of Hematopoietic Cell Transplantation (HCT): CIBMTR Summary Slides. Available at https://wwwcibmtrorg. 2018.

24. Markey KA, MacDonald KPA, Hill GR. The biology of graft-versus-host disease: experimental systems instructing clinical practice. Blood. 2014;124(3):354-62.

25. Terwey TH, Le Duc TM, Hemmati PG, le Coutre P, Nagy M, Martus P, et al. NIH-defined graft-versus-host disease and evidence for a potent graft-versus-leukemia effect in patients with acute lymphoblastic leukemia. Annals of Oncology. 2013;24(5):1363-70.

26. Pidala J, Vogelsang G, Martin P, Chai X, Storer B, Pavletic S, et al. Overlap subtype of chronic graft-versus-host disease is associated with an adverse prognosis, functional impairment, and inferior patient-reported outcomes: a Chronic Graft-versus-Host Disease Consortium study. Haematologica. 2012;97(3):451-8.

27. Thomas ED SR, Clift RA, Fefer A, Johnson L, Neiman PE, Lerner KG, Glucksberg H, Buckner CD. Bone-marrow transplantation. N Engl J Med. 1975;292(17):8.

28. Glucksberg H SR, Fefer A, Buckner CD, Neiman PE, Clift RA, Lerner KG, Thomas ED. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation. 1974;18(4):195-304.

29. Cahn J-Y, Klein JP, Lee SJ, Milpied N, Blaise D, Antin JH, et al. Prospective evaluation of 2 acute graft-versus-host (GVHD) grading systems: a joint Société Française de Greffe de Moëlle et Thérapie Cellulaire (SFGM-TC), Dana Farber Cancer Institute (DFCI), and International Bone Marrow Transplant Registry (IBMTR) prospective study. Blood. 2005;106(4):1495-500.

30. Greinix HT, Eikema D-J, Koster L, Penack O, Yakoub-Agha I, Montoto S, et al. Incidence of Acute Graft-Versus-Host Disease and Survival after Allogeneic Hematopoietic Cell Transplantation over Time: A Study from the Transplant Complications and Chronic Malignancies Working Party of the EBMT. Blood. 2018;132(Supplement 1):2120-.

31. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. Nature Reviews Immunology. 2012;12(6):443-58.

32. Arai S, Arora M, Wang T, Spellman SR, He W, Couriel DR, et al. Increasing incidence of chronic graft-versus-host disease in allogeneic transplantation: a report from the Center for International Blood and Marrow Transplant Research. Biol Blood Marrow Transplant. 2015;21(2):266-74.

33. Socié G. Chronic GVHD: a new risk score? Blood. 2011;117(24):6408-9.

34. Sarantopoulos S, Ritz J. Aberrant B-cell homeostasis in chronic GVHD. Blood. 2015;125(11):1703-7.

35. Young JS, Wu T, Chen Y, Zhao D, Liu H, Yi T, et al. Donor B cells in transplants augment clonal expansion and survival of pathogenic CD4+ T cells that mediate autoimmune-like chronic graft-versus-host disease. J Immunol. 2012;189(1):222-33.

36. Kuzmina Z, Krenn K, Petkov V, Körmöczi U, Weigl R, Rottal A, et al. CD19+CD21low B cells and patients at risk for NIH-defined chronic graft-versus-host disease with bronchiolitis obliterans syndrome. Blood. 2013;121(10):1886-95.

37. Zhao Y, He R, Oerther S, Zhou W, Vosough M, Hassan M. Cardiovascular Complications in Hematopoietic Stem Cell Transplanted Patients. Journal of Personalized Medicine [Internet]. 2022; 12(11).

38. Tonorezos ES, Stillwell EE, Calloway JJ, Glew T, Wessler JD, Rebolledo BJ, et al. Arrhythmias in the setting of hematopoietic cell transplants. Bone Marrow Transplantation. 2015;50(9):1212-6.

39. Versluys AB, Grotenhuis HB, Boelens MJJ, Mavinkurve-Groothuis AMC, Breur J. Predictors and Outcome of Pericardial Effusion After Hematopoietic Stem Cell Transplantation in Children. Pediatric cardiology. 2018;39(2):236-44.

40. Alblooshi R, Kanfar S, Lord B, Atenafu EG, Michelis FV, Pasic I, et al. Clinical prevalence and outcome of cardiovascular events in the first 100 days postallogeneic hematopoietic stem cell transplant. European Journal of Haematology. 2021;106(1):32-9.

41. Scott JM, Armenian S, Giralt S, Moslehi J, Wang T, Jones LW. Cardiovascular disease following hematopoietic stem cell transplantation: Pathogenesis, detection, and the cardioprotective role of aerobic training. Crit Rev Oncol Hematol. 2016;98:222-34.

42. Armenian SH, Sun C-L, Vase T, Ness KK, Blum E, Francisco L, et al. Cardiovascular risk factors in hematopoietic cell transplantation survivors: role in development of subsequent cardiovascular disease. Blood. 2012;120(23):4505-12.

43. Tichelli A, Bucher C, Rovó A, Stussi G, Stern M, Paulussen M, et al. Premature cardiovascular disease after allogeneic hematopoietic stem-cell transplantation. Blood. 2007;110(9):3463-71.

44. Goldberg MA, Antin JH, Guinan EC, Rappeport JM. Cyclophosphamide cardiotoxicity: an analysis of dosing as a risk factor. Blood. 1986;68(5):1114-8.

45. Duléry R, Mohty R, Labopin M, Sestili S, Malard F, Brissot E, et al. Early Cardiac Toxicity Associated With Post-Transplant Cyclophosphamide in Allogeneic Stem Cell Transplantation. JACC: CardioOncology. 2021;3(2):250-9.

46. Bacigalupo A, Milone G, Cupri A, Severino A, Fagioli F, Berger M, et al. Steroid treatment of acute graft-versus-host disease grade I: a randomized trial. Haematologica. 2017;102(12):2125-33.

47. Lee HJ, Oran B, Saliba RM, Couriel DM, Shin K, Massey P, et al. Steroid myopathy in patients with acute graft-versus-host disease treated with high-dose steroid therapy. Bone Marrow Transplantation. 2006;38(4):299-303.

48. Malard F, Huang X-J, Sim JPY. Treatment and unmet needs in steroid-refractory acute graft-versus-host disease. Leukemia. 2020;34(5):1229-40.

49. Nishimoto M, Nakamae H, Koh H, Nakamae M, Hirose A, Hayashi Y, et al. Response-guided therapy for steroid-refractory acute GVHD starting with very-low-dose antithymocyte globulin. Experimental hematology. 2015;43(3):177-9.

50. Yalniz FF, Hefazi M, McCullough K, Litzow MR, Hogan WJ, Wolf R, et al. Safety and Efficacy of Infliximab Therapy in the Setting of Steroid-Refractory Acute Graft-versus-Host Disease. Biology of Blood and Marrow Transplantation. 2017;23(9):1478-84.

51. Nygaard M, Andersen NS, Moser CE, Olesen G, Schjødt IM, Heilmann C, et al. Evaluation of infliximab as second-line treatment of acute graft versus host disease - validating response on day 7 and 28 as predictors of survival. Bone Marrow Transplantation. 2018;53(7):844-51.

52. García-Cadenas I, Rivera I, Martino R, Esquirol A, Barba P, Novelli S, et al. Patterns of infection and infection-related mortality in patients with steroid-refractory acute graft versus host disease. Bone Marrow Transplantation. 2017;52(1):107-13.

53. Chakupurakal G, García-Márquez MA, Shimabukuro-Vornhagen A, Theurich S, Holtick U, Hallek M, et al. Immunological effects in patients with steroid-refractory graft-versus-host disease following treatment with basiliximab, a CD25 monoclonal antibody. Eur J Haematol. 2016;97(2):121-7.

54. Busca A, Saroglia EM, Lanino E, Manfredini L, Uderzo C, Nicolini B, et al. Mycophenolate mofetil (MMF) as therapy for refractory chronic GVHD (cGVHD) in children receiving bone marrow transplantation. Bone Marrow Transplant. 2000;25(10):1067-71.

55. Modemann F, Ayuk F, Wolschke C, Christopeit M, Janson D, von Pein U-M, et al. Ruxolitinib plus extracorporeal photopheresis (ECP) for steroid refractory acute graft-versus-host disease of lower GI-tract after allogeneic stem cell transplantation leads to increased regulatory T cell level. Bone Marrow Transplantation. 2020;55(12):2286-93.

56. Mankarious M, Matthews NC, Snowden JA, Alfred A. Extracorporeal Photopheresis (ECP) and the Potential of Novel Biomarkers in Optimizing Management of Acute and Chronic Graft vs. Host Disease (GvHD). 2020;11.

57. Kelly K, Rasko JEJ. Mesenchymal Stromal Cells for the Treatment of Graft Versus Host Disease. 2021;12.

58. Ragon BK, Mehta RS, Gulbis AM, Saliba RM, Chen J, Rondon G, et al. Pentostatin therapy for steroid-refractory acute graft versus host disease: identifying those who may benefit. Bone Marrow Transplant. 2018;53(3):315-25.

59. Martini DJ, Chen Y-B, DeFilipp Z. Recent FDA Approvals in the Treatment of Graft-Versus-Host Disease. The Oncologist. 2022;27(8):685-93.

60. Jagasia M, Perales M-A, Schroeder MA, Ali H, Shah NN, Chen Y-B, et al. Results from REACH1, a Single-Arm Phase 2 Study of Ruxolitinib in Combination with Corticosteroids for the Treatment of Steroid-Refractory Acute Graft-Vs-Host Disease. Blood. 2018;132(Supplement 1):601-.

61. Zeiser R, von Bubnoff N, Butler J, Mohty M, Niederwieser D, Or R, et al. Ruxolitinib for Glucocorticoid-Refractory Acute Graft-versus-Host Disease. New England Journal of Medicine. 2020;382(19):1800-10.

62. Gupta V, Kumar Singh S, Agrawal V, Bali Singh T. Role of ACE inhibitors in anthracycline-induced cardiotoxicity: A randomized, double-blind, placebo-controlled trial. Pediatric blood & cancer. 2018;65(11):e27308.

63. Bosch X, Rovira M, Sitges M, Domènech A, Ortiz-Pérez JT, de Caralt TM, et al. Enalapril and carvedilol for preventing chemotherapy-induced left ventricular systolic dysfunction in patients with malignant hemopathies: the OVERCOME trial (preventiOn of left Ventricular dysfunction with Enalapril and caRvedilol in patients submitted to intensive ChemOtherapy for the treatment of Malignant hEmopathies). Journal of the American College of Cardiology. 2013;61(23):2355-62.

64. Singla A, Hogan WJ, Ansell SM, Buadi FK, Dingli D, Dispenzieri A, et al. Incidence of supraventricular arrhythmias during autologous peripheral blood stem cell transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2013;19(8):1233-7.

65. Hamilton BK. Current approaches to prevent and treat GVHD after allogeneic stem cell transplantation. Hematology. 2018;2018(1):228-35.

66. Gooptu M, Antin JH. GVHD Prophylaxis 2020. Frontiers in immunology. 2021;12:605726.

67. Storb R, Deeg HJ, Whitehead J, Appelbaum F, Beatty P, Bensinger W, et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. The New England journal of medicine. 1986;314(12):729-35.

68. Storb R, Antin JH, Cutler C. Should methotrexate plus calcineurin inhibitors be considered standard of care for prophylaxis of acute graft-versus-host disease? Biol Blood Marrow Transplant. 2010;16(1 Suppl):S18-S27.

69. Sandmaier BM, Kornblit B, Storer BE, Olesen G, Maris MB, Langston AA, et al. Addition of sirolimus to standard cyclosporine plus mycophenolate mofetil-based graft-versus-host disease prophylaxis for patients after unrelated non-myeloablative haemopoietic stem cell transplantation: a multicentre, randomised, phase 3 trial. The Lancet Haematology. 2019;6(8):e409-e18.

70. Cutler C, Stevenson K, Kim HT, Richardson P, Ho VT, Linden E, et al. Sirolimus is associated with veno-occlusive disease of the liver after myeloablative allogeneic stem cell transplantation. Blood. 2008;112(12):4425-31.

71. Namdaroglu S, Kaya AH, Batgi H, Kayikci O, Dal MS, Iskender D, et al. Impacts of post-transplantation cyclophosphamide treatment after allogeneic hematopoietic stem cell transplantation in acute myeloid leukemia. Scientific Reports. 2019;9(1):2046.

72. Al-Homsi AS, Roy TS, Cole K, Feng Y, Duffner U. Post-Transplant High-Dose Cyclophosphamide for the Prevention of Graft-versus-Host Disease. Biology of Blood and Marrow Transplantation. 2015;21(4):604-11.

73. Strauss G, Osen W, Debatin KM. Induction of apoptosis and modulation of activation and effector function in T cells by immunosuppressive drugs. Clin Exp Immunol. 2002;128(2):255-66.

74. Nunes NS, Kanakry CG. Mechanisms of Graft-versus-Host Disease Prevention by Post-transplantation Cyclophosphamide: An Evolving Understanding. Frontiers in Immunology. 2019;10(2668).

75. Battipaglia G, Labopin M, Kröger N, Vitek A, Afanasyev B, Hilgendorf I, et al. Posttransplant cyclophosphamide vs antithymocyte globulin in HLA-mismatched unrelated donor transplantation. Blood. 2019;134(11):892-9.

76. Kwon M, Bailén R, Pascual-Cascón MJ, Gallardo-Morillo AI, García Sola A, Balsalobre P, et al. Posttransplant cyclophosphamide vs cyclosporin A and methotrexate as GVHD prophylaxis in matched sibling transplantation. Blood Advances. 2019;3(21):3351-9.

77. Barba P, Hilden P, Devlin SM, Maloy M, Dierov D, Nieves J, et al. Ex Vivo CD34+–Selected T Cell–Depleted Peripheral Blood Stem Cell Grafts for Allogeneic Hematopoietic Stem Cell Transplantation in Acute Leukemia and Myelodysplastic Syndrome Is Associated with Low Incidence of Acute and Chronic Graft-versus-Host Disease and High Treatment Response. Biology of Blood and Marrow Transplantation. 2017;23(3):452-8.

78. Seif AE, Li Y, Aplenc R, Barrett DM, Casper JT, Freedman JL, et al. Favorable Chronic Graft-Versus-Host-Disease (GVHD), Event-Free (EFS), and Overall Survival (OS) Rates Following Partially CD3-Depleted Alternative Donor Peripheral Stem Cell Transplantation (PSCT) for Pediatric Hematologic Malignancies. Biology of Blood and Marrow Transplantation. 2016;22(3):S62-S3.

79. Rådestad E, Sundin M, Törlén J, Thunberg S, Önfelt B, Ljungman P, et al. Individualization of Hematopoietic Stem Cell Transplantation Using Alpha/Beta T-Cell Depletion. Frontiers in Immunology. 2019;10:189-.

80. Fredricks DN. The gut microbiota and graft-versus-host disease. J Clin Invest. 2019;129(5):1808-17.

81. Moy RH, Huffman AP, Richman LP, Crisalli L, Wang XK, Hoxie JA, et al. Clinical and immunologic impact of CCR5 blockade in graft-versus-host disease prophylaxis. Blood. 2017;129(7):906-16.

82. Martin PJ. Bortezomib for prevention of acute graft-versus-host disease: a conclusion reached. Haematologica. 2018;103(3):377-9.

83. Choi SW, Braun T, Henig I, Gatza E, Magenau J, Parkin B, et al. Vorinostat plus tacrolimus/methotrexate to prevent GVHD after myeloablative conditioning, unrelated donor HCT. Blood. 2017;130(15):1760-7.

84. Farag SS, Abu Zaid M, Schwartz JE, Thakrar TC, Blakley AJ, Abonour R, et al. Dipeptidyl Peptidase 4 Inhibition for Prophylaxis of Acute Graft-versus-Host Disease. New England Journal of Medicine. 2021;384(1):11-9.

85. Godoy JAP, Paiva RMA, Souza AM, Kondo AT, Kutner JM, Okamoto OK. Clinical Translation of Mesenchymal Stromal Cell Therapy for Graft Versus Host Disease. Frontiers in Cell and Developmental Biology. 2019;7(255).

86. FDA. FDA approves abatacept for prophylaxis of acute graft versus host disease: https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-abatacept-prophylaxis-acute-graft-versus-host-disease; 2021 [

87. Burchert A, Bug G, Fritz LV, Finke J, Stelljes M, Röllig C, et al. Sorafenib Maintenance After Allogeneic Hematopoietic Stem Cell Transplantation for Acute Myeloid Leukemia With FLT3–Internal Tandem Duplication Mutation (SORMAIN). Journal of Clinical Oncology. 2020;38(26):2993-3002.

88. Schmidinger M, Zielinski CC, Vogl UM, Bojic A, Bojic M, Schukro C, et al. Cardiac Toxicity of Sunitinib and Sorafenib in Patients With Metastatic Renal Cell Carcinoma. Journal of Clinical Oncology. 2008;26(32):5204-12.

89. Majhail NS, Rizzo JD, Lee SJ, Aljurf M, Atsuta Y, Bonfim C, et al. Recommended screening and preventive practices for long-term survivors after hematopoietic cell transplantation. Bone Marrow Transplantation. 2012;47(3):337-41.

90. Ilkan Z, Akar FG. The Mitochondrial Translocator Protein and the Emerging Link Between Oxidative Stress and Arrhythmias in the Diabetic Heart. Front Physiol. 2018;9:1518.

91. Ebrahimi S, Soltani A, Hashemy SI. Oxidative stress in cervical cancer pathogenesis and resistance to therapy. J Cell Biochem. 2018.

92. Schwab L, Goroncy L, Palaniyandi S, Gautam S, Triantafyllopoulou A, Mocsai A, et al. Neutrophil granulocytes recruited upon translocation of intestinal bacteria enhance graft-versus-host disease via tissue damage. Nature Medicine. 2014;20(6):648-54.

93. Sari I, Cetin A, Kaynar L, Saraymen R, Hacioglu SK, Ozturk A, et al. Disturbance of Pro-oxidative/Antioxidative Balance in Allogeneic Peripheral Blood Stem Cell Transplantation. Annals of Clinical & Laboratory Science. 2008;38(2):120-5.

94. Suh JH, Kanathezhath B, Shenvi S, Guo H, Zhou A, Tiwana A, et al. Thiol/redox metabolomic profiling implicates GSH dysregulation in early experimental graft versus host disease (GVHD). PLoS One. 2014;9(2):e88868-e.

95. Sofi MH, Wu Y, Dai M, Schutt SD, Daenthanasanmak A, Heinrichs JL, et al. Oxidative Stress Regulates T Cell Pathogenicity in Gvhd. Blood. 2017;130(Supplement 1):3168-.

96. Im K-I, Kim N, Lim J-Y, Nam Y-S, Lee E-S, Kim E-J, et al. The Free Radical Scavenger NecroX-7 Attenuates Acute Graft-versus-Host Disease via Reciprocal Regulation of Th1/Regulatory T Cells and Inhibition of HMGB1 Release. J Immunol. 2015;194(11):5223-32.

97. Sofi MH, Wu Y, Schutt SD, Dai M, Daenthanasanmak A, Heinrichs Voss J, et al. Thioredoxin-1 confines T cell alloresponse and pathogenicity in graft-versus-host disease. J Clin Invest. 2019;129(8):2760-74.

98. Rezende BM, Bernardes PTT, Gonçalves WA, de Resende CB, Athayde RM, Ávila TV, et al. Treatment with Apocynin Limits the Development of Acute Graft-versus-Host Disease in Mice. J Immunol Res. 2019;2019:9015292-.

99. Toubai T, Tamaki H, Peltier DC, Rossi C, Oravecz-Wilson K, Liu C, et al. Mitochondrial Deacetylase SIRT3 Plays an Important Role in Donor T Cell Responses after Experimental Allogeneic Hematopoietic Transplantation. J Immunol. 2018;201(11):3443-55.

100. Han J, Ma S, Gong H, Liu S, Lei L, Hu B, et al. Inhibition of Acute Graft-versus-Host Disease with Retention of Graft-versus-Tumor Effects by Dimethyl Fumarate. Frontiers in immunology. 2017;8:1605.

101. Yi T, Li X, Wang E, Zhang Y, Fu Y, Li J, et al. Activation of the Nuclear Erythroid 2-Related Factor 2 Antioxidant Responsive Element (Nrf2-ARE) Signaling Pathway Alleviates Acute Graft-Versus-Host Disease by Reducing Oxidative Stress and Inhibiting Infiltration of Inflammatory Cells in an Allogeneic Stem Cell Transplantation Mouse Model. Medical science monitor : international medical journal of experimental and clinical research. 2018;24:5973-9.

102. Han J, Ma S, Gong H, Liu S, Lei L, Hu B, et al. Inhibition of Acute Graft-versus-Host Disease with Retention of Graft-versus-Tumor Effects by Dimethyl Fumarate. Frontiers in Immunology. 2017;8(1605).

103. Ayza MA, Zewdie KA, Tesfaye BA, Wondafrash DZ, Berhe AH. The Role of Antioxidants in Ameliorating Cyclophosphamide-Induced Cardiotoxicity. Oxidative Medicine and Cellular Longevity. 2020;2020:4965171.

104. Mythili Y, Sudharsan PT, Sudhahar V, Varalakshmi P. Protective effect of dl-α-lipoic acid on cyclophosphamide induced hyperlipidemic cardiomyopathy. European Journal of Pharmacology. 2006;543(1):92-6.

105. Gunes S, Sahinturk V, Karasati P, Sahin IK, Ayhanci A. Cardioprotective Effect of Selenium Against Cyclophosphamide-Induced Cardiotoxicity in Rats. Biological trace element research. 2017;177(1):107-14.

106. Refaie MMM, Shehata S, El-Hussieny M, Abdelraheem WM, Bayoumi AMA. Role of ATP-Sensitive Potassium Channel (K(ATP)) and eNOS in Mediating the Protective Effect of Nicorandil in Cyclophosphamide-Induced Cardiotoxicity. Cardiovascular toxicology. 2020;20(1):71-81.

107. El-Sheikh AA, Abdelzaher WY, Gad AA, Abdel-Gaber SA. Purine versus non-purine xanthine oxidase inhibitors against cyclophosphamide-induced cardiac and bone marrow toxicity in rats. Human & experimental toxicology. 2020;39(3):249-61.

108. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature. 1997;390(6655):45-51.

109. Hanson K, Fisher K, Hooper NM. Exploiting the neuroprotective effects of α-klotho to tackle ageing- and neurodegeneration-related cognitive dysfunction. Neuronal signaling. 2021;5(2):Ns20200101.

110. Kuro-o M. The Klotho proteins in health and disease. Nature Reviews Nephrology. 2019;15(1):27-44.

111. Kim JH, Hwang KH, Park KS, Kong ID, Cha SK. Biological Role of Anti-aging Protein Klotho. Journal of lifestyle medicine. 2015;5(1):1-6.

112. Chen CD, Podvin S, Gillespie E, Leeman SE, Abraham CR. Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(50):19796-801.

113. Lu X, Hu MC. Klotho/FGF23 Axis in Chronic Kidney Disease and Cardiovascular Disease. Kidney Diseases. 2017;3(1):15-23.

114. Rossaint J, Unruh M, Zarbock A. Fibroblast growth factor 23 actions in inflammation: a key factor in CKD outcomes. Nephrology Dialysis Transplantation. 2017;32(9):1448-53.

115. Krick S, Grabner A, Baumlin N, Yanucil C, Helton S, Grosche A, et al. Fibroblast growth factor 23 and Klotho contribute to airway inflammation. European Respiratory Journal. 2018;52(1):1800236.

116. El-Hodhod MA-A, Hamdy AM, Abbas AA, Moftah SG, Ramadan AAM. Fibroblast growth factor 23 contributes to diminished bone mineral density in childhood inflammatory bowel disease. BMC Gastroenterology. 2012;12(1):44.

117. Nagai T, Yamada K, Kim HC, Kim YS, Noda Y, Imura A, et al. Cognition impairment in the genetic model of aging klotho gene mutant mice: a role of oxidative stress. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2003;17(1):50-2.

118. Brobey RK, German D, Sonsalla PK, Gurnani P, Pastor J, Hsieh CC, et al. Klotho Protects Dopaminergic Neuron Oxidant-Induced Degeneration by Modulating ASK1 and p38 MAPK Signaling Pathways. PLoS One. 2015;10(10):e0139914.

119. Wang Y, Kuro-o M, Sun Z. Klotho gene delivery suppresses Nox2 expression and attenuates oxidative stress in rat aortic smooth muscle cells via the cAMP-PKA pathway. Aging cell. 2012;11(3):410-7.

120. Villeneuve E, Gosselin S. N-Acetylcysteine. In: Brent J, Burkhart K, Dargan P, Hatten B, Megarbane B, Palmer R, et al., editors. Critical Care Toxicology: Diagnosis and Management of the Critically Poisoned Patient. Cham: Springer International Publishing; 2017. p. 2879-88.

121. Aparicio-Trejo OE, Reyes-Fermín LM, Briones-Herrera A, Tapia E, León-Contreras JC, Hernández-Pando R, et al. Protective effects of N-acetyl-cysteine in mitochondria bioenergetics, oxidative stress, dynamics and S-glutathionylation alterations in acute kidney damage induced by folic acid. Free Radical Biology and Medicine. 2019;130:379-96.

122. El-Serafi I, Remberger M, El-Serafi A, Benkessou F, Zheng W, Martell E, et al. The effect of N-acetyl-l-cysteine (NAC) on liver toxicity and clinical outcome after hematopoietic stem cell transplantation. Scientific Reports. 2018;8(1):8293.

123. Abdel-Fattah MM, Salama AAA, Messiha BAS. Immunomodulatory and anti-inflammatory effects of N-acetylcysteine in ovalbumin-sensitized rats. Beni-Suef University Journal of Basic and Applied Sciences. 2022;11(1):2.

124. Cazzola M, Calzetta L, Page C, Jardim J, Chuchalin AG, Rogliani P, et al. Influence of N-acetylcysteine on chronic bronchitis or COPD exacerbations: a meta-analysis. Eur Respir Rev. 2015;24(137):451-61.

125. Kiuru A, Ahola T, Klenberg L, Tommiska V, Lano A, Kleemola P, et al. Postnatal N-acetylcysteine does not provide neuroprotection in extremely low birth weight infants: A follow-up of a randomized controlled trial. Early Human Development. 2019;132:13-7.

126. Karlsson H, Nava S, Remberger M, Hassan Z, Hassan M, Ringden O. N-acetyl-L-cysteine increases acute graft-versus-host disease and promotes T-cell-mediated immunity in vitro. European journal of immunology. 2011;41(4):1143-53.

127. He R, Zheng W, Ginman T, Ottosson H, Norgren S, Zhao Y, et al. Pharmacokinetic profile of N-acetylcysteine amide and its main metabolite in mice using new analytical method. European Journal of Pharmaceutical Sciences. 2020;143:105158.

128. Goyal V, Bews H, Cheung D, Premecz S, Mandal S, Shaikh B, et al. The Cardioprotective Role of N-Acetyl Cysteine Amide in the Prevention of Doxorubicin and Trastuzumab-Mediated Cardiac Dysfunction. Can J Cardiol. 2016;32(12):1513-9.

129. Kawoos U, Abutarboush R, Zarriello S, Qadri A, Ahlers ST, McCarron RM, et al. N-acetylcysteine Amide Ameliorates Blast-Induced Changes in Blood-Brain Barrier Integrity in Rats. Front Neurol. 2019;10:650-.

130. Maddirala Y, Tobwala S, Karacal H, Ercal N. Prevention and reversal of selenite-induced cataracts by N-acetylcysteine amide in Wistar rats. BMC Ophthalmol. 2017;17(1):54.

131. Atlas D. Emerging therapeutic opportunities of novel thiol-amides, NAC-amide (AD4/NACA) and thioredoxin mimetics (TXM-Peptides) for neurodegenerative-related disorders. Free Radical Biology and Medicine. 2021;176:120-41.

132. Wu W, Goldstein G, Adams C, Matthews RH, Ercal N. Separation and quantification of N-acetyl-l-cysteine and N-acetyl-cysteine-amide by HPLC with fluorescence detection. Biomed Chromatogr. 2006;20(5):415-22.

133. King B, Vance J, Wall GM, Shoup R. Quantitation of free and total N-acetylcysteine amide and its metabolite N-acetylcysteine in human plasma using derivatization and electrospray LC-MS/MS. Journal of Chromatography B. 2019;1109:25-36.

134. Cooke KR, Kobzik L, Martin TR, Brewer J, Delmonte J, Jr., Crawford JM, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. Blood. 1996;88(8):3230-9.

135. Hu MC, Moe OW. Klotho as a potential biomarker and therapy for acute kidney injury. Nature reviews Nephrology. 2012;8(7):423-9.

136. Henden AS, Hill GR. Cytokines in Graft-versus-Host Disease. Journal of immunology (Baltimore, Md : 1950). 2015;194(10):4604-12.

137. Lin Y, Zeng B, Wang Y, Chen Y, Fang Z, Zhang J, et al. Unpaired Multi-Domain Stain Transfer for Kidney Histopathological Images. Proceedings of the AAAI Conference on Artificial Intelligence. 2022;36(2):1630-7.

138. Na IK, Markley JC, Tsai JJ, Yim NL, Beattie BJ, Klose AD, et al. Concurrent visualization of trafficking, expansion, and activation of T lymphocytes and T-cell precursors in vivo. Blood. 2010;116(11):e18-25.

139. Beilhack A, Schulz S, Baker J, Beilhack GF, Nishimura R, Baker EM, et al. Prevention of acute graft-versus-host disease by blocking T-cell entry to secondary lymphoid organs. Blood. 2008;111(5):2919-28.

140. Riesner K, Shi Y, Jacobi A, Kräter M, Kalupa M, McGearey A, et al. Initiation of acute graft-versus-host disease by angiogenesis. Blood. 2017;129(14):2021-32.

141. Wysocki CA, Panoskaltsis-Mortari A, Blazar BR, Serody JS. Leukocyte migration and graft-versus-host disease. Blood. 2005;105(11):4191-9.

142. Habas K, Shang L. Alterations in intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in human endothelial cells. Tissue & cell. 2018;54:139-43.

143. Kofler NM, Shawber CJ, Kangsamaksin T, Reed HO, Galatioto J, Kitajewski J. Notch signaling in developmental and tumor angiogenesis. Genes & cancer. 2011;2(12):1106-16.

144. Kanduri SR, Cheungpasitporn W, Thongprayoon C, Bathini T, Kovvuru K, Garla V, et al. Incidence and mortality of acute kidney injury in patients undergoing hematopoietic stem cell transplantation: a systematic review and meta-analysis. QJM : monthly journal of the Association of Physicians. 2020;113(9):621-32.

145. Miyata M, Ichikawa K, Matsuki E, Watanabe M, Peltier D, Toubai T. Recent Advances of Acute Kidney Injury in Hematopoietic Cell Transplantation. 2022;12.

146. Bolignano D, Donato V, Coppolino G, Campo S, Buemi A, Lacquaniti A, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a marker of kidney damage. American journal of kidney diseases : the official journal of the National Kidney Foundation. 2008;52(3):595-605.

147. Devarajan P. Neutrophil gelatinase-associated lipocalin: a promising biomarker for human acute kidney injury. Biomarkers in medicine. 2010;4(2):265-80.

148. Törnblom S, Nisula S, Petäjä L, Vaara ST, Haapio M, Pesonen E, et al. Urine NGAL as a biomarker for septic AKI: a critical appraisal of clinical utility—data from the observational FINNAKI study. Annals of Intensive Care. 2020;10(1):51.

149. Ghadially FN. 7 - Lysosomes. In: Ghadially FN, editor. Ultrastructural Pathology of the Cell and Matrix (Third Edition): Butterworth-Heinemann; 1988. p. 589-765.

150. Surendran K, Vitiello SP, Pearce DA. Lysosome dysfunction in the pathogenesis of kidney diseases. Pediatric nephrology (Berlin, Germany). 2014;29(12):2253-61.

151. Mori K, Lee HT, Rapoport D, Drexler IR, Foster K, Yang J, et al. Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. The Journal of clinical investigation. 2005;115(3):610-21.

152. Sugiura H, Yoshida T, Tsuchiya K, Mitobe M, Nishimura S, Shirota S, et al. Klotho reduces apoptosis in experimental ischaemic acute renal failure. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2005;20(12):2636-45.

153. Hu MC, Shi M, Zhang J, Quiñones H, Kuro-o M, Moe OW. Klotho deficiency is an early biomarker of renal ischemia-reperfusion injury and its replacement is protective. Kidney international. 2010;78(12):1240-51.

154. Shi M, Flores B, Gillings N, Bian A, Cho HJ, Yan S, et al. αKlotho Mitigates Progression of AKI to CKD through Activation of Autophagy. 2016;27(8):2331-45.

155. Neyra JA, Hu MC, Moe OW. Klotho in Clinical Nephrology. Diagnostic and Therapeutic Implications. 2021;16(1):162-76.

156. Eghtedari Y, Oh LJ, Girolamo ND, Watson SL. The role of topical N-acetylcysteine in ocular therapeutics. Survey of Ophthalmology. 2022;67(2):608-22.

157. Gaucher C, Boudier A, Bonetti J, Clarot I, Leroy P, Parent M. Glutathione: Antioxidant Properties Dedicated to Nanotechnologies. Antioxidants (Basel, Switzerland). 2018;7(5).

158. Chatterjee S. Chapter Two - Oxidative Stress, Inflammation, and Disease. In: Dziubla T, Butterfield DA, editors. Oxidative Stress and Biomaterials: Academic Press; 2016. p. 35-58.

159. Lugrin J, Rosenblatt-Velin N, Parapanov R, Liaudet L. The role of oxidative stress during inflammatory processes. 2014;395(2):203-30.

160. Sun HJ, Wu ZY, Nie XW, Bian JS. Role of Endothelial Dysfunction in Cardiovascular Diseases: The Link Between Inflammation and Hydrogen Sulfide. Frontiers in pharmacology. 2019;10:1568.

161. Bui TM, Wiesolek HL, Sumagin R. ICAM-1: A master regulator of cellular responses in inflammation, injury resolution, and tumorigenesis. Journal of leukocyte biology. 2020;108(3):787-99.

162. Dirven HA, van Ommen B, van Bladeren PJ. Involvement of human glutathione S-transferase isoenzymes in the conjugation of cyclophosphamide metabolites with glutathione. Cancer Res. 1994;54(23):6215-20.

163. Cai H, Harrison DG. Endothelial Dysfunction in Cardiovascular Diseases: The Role of Oxidant Stress. Circulation Research. 2000;87(10):840-4.

164. Pernow J, Jung C. Arginase as a potential target in the treatment of cardiovascular disease: reversal of arginine steal? Cardiovascular research. 2013;98(3):334-43.

165. Sewduth R, Santoro MM. "Decoding" Angiogenesis: New Facets Controlling Endothelial Cell Behavior. Frontiers in physiology. 2016;7:306.

166. CS R. NACA for the Treatment of Chronic or Low Impact Brian Trauma. Google Patents US 2015112715 A1. 2015.