

# **Senolytics dasatinib and quercetin in metabolic dysfunction-associated steatohepatitis: a proof-of-principle randomized, controlled trial**

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In the format provided by the  
authors and unedited

Fig. S1. Study design

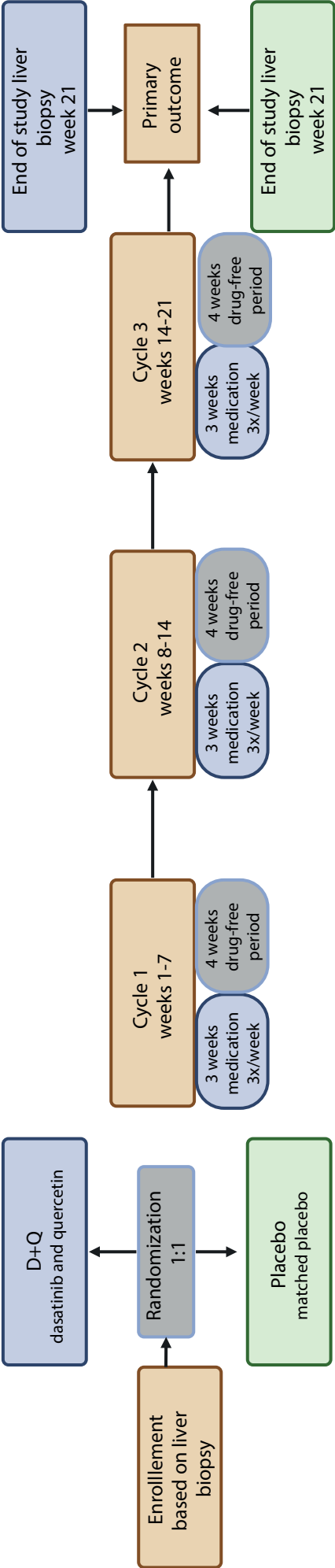


Fig. S2. Marker gene expression across cell types

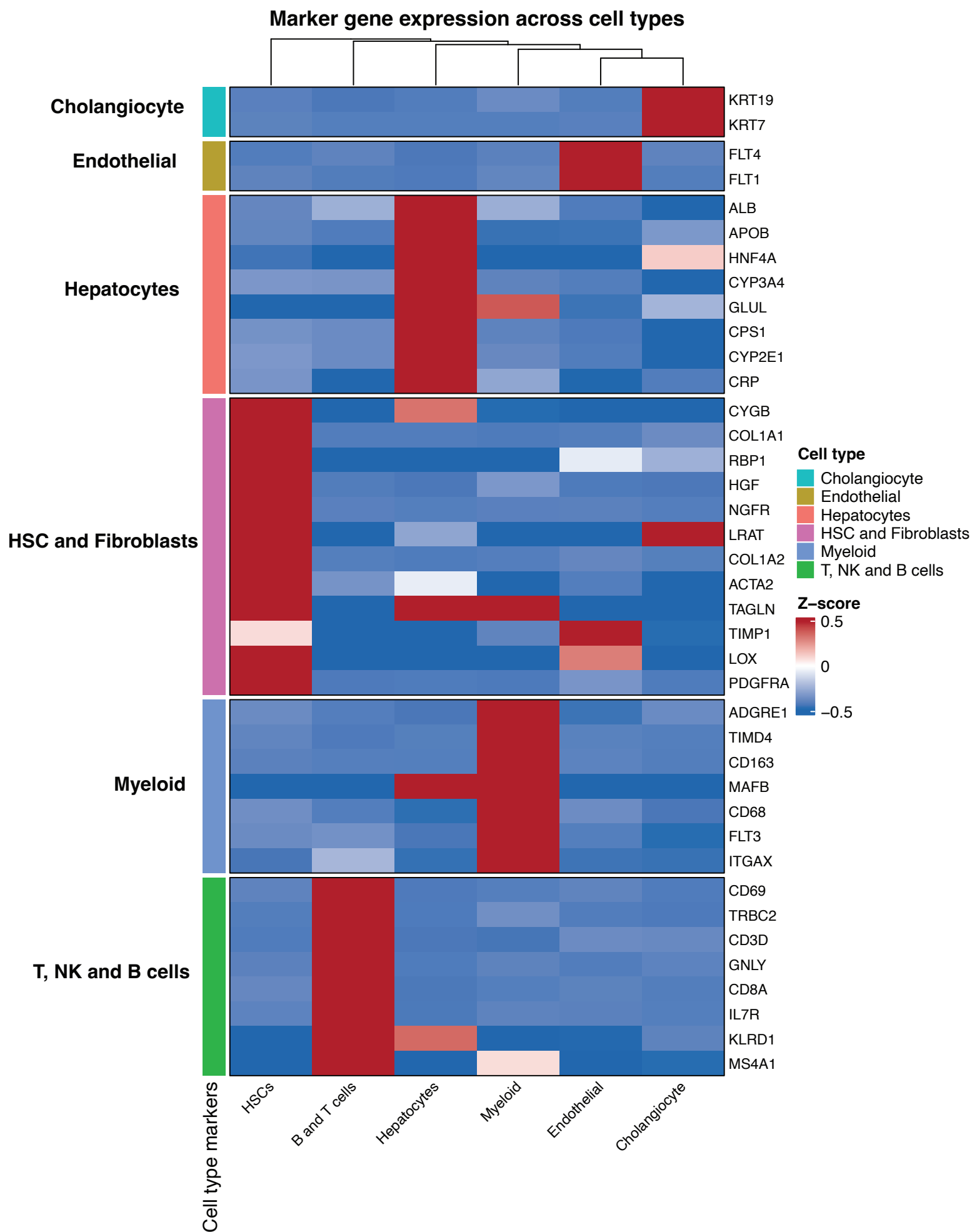
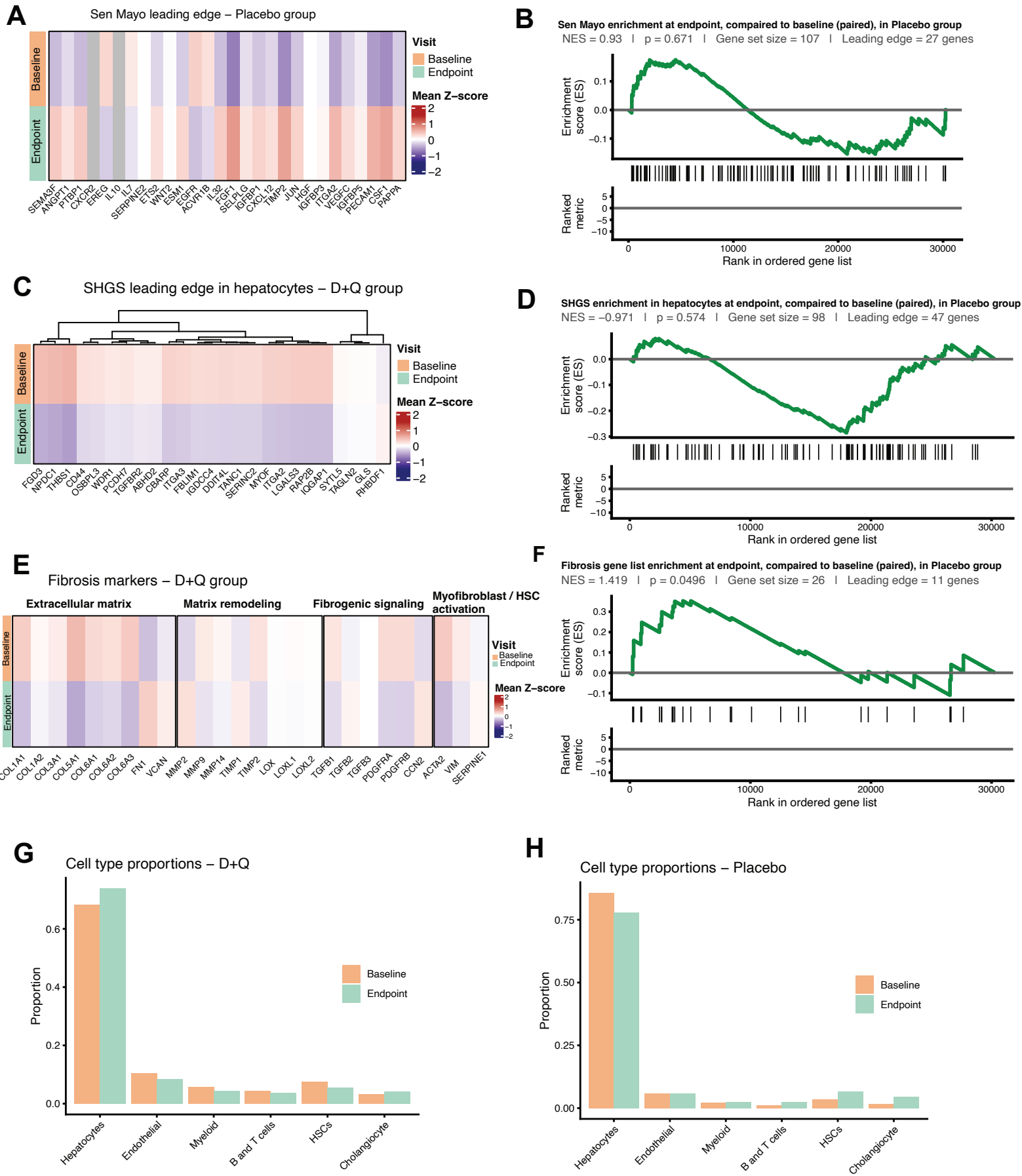
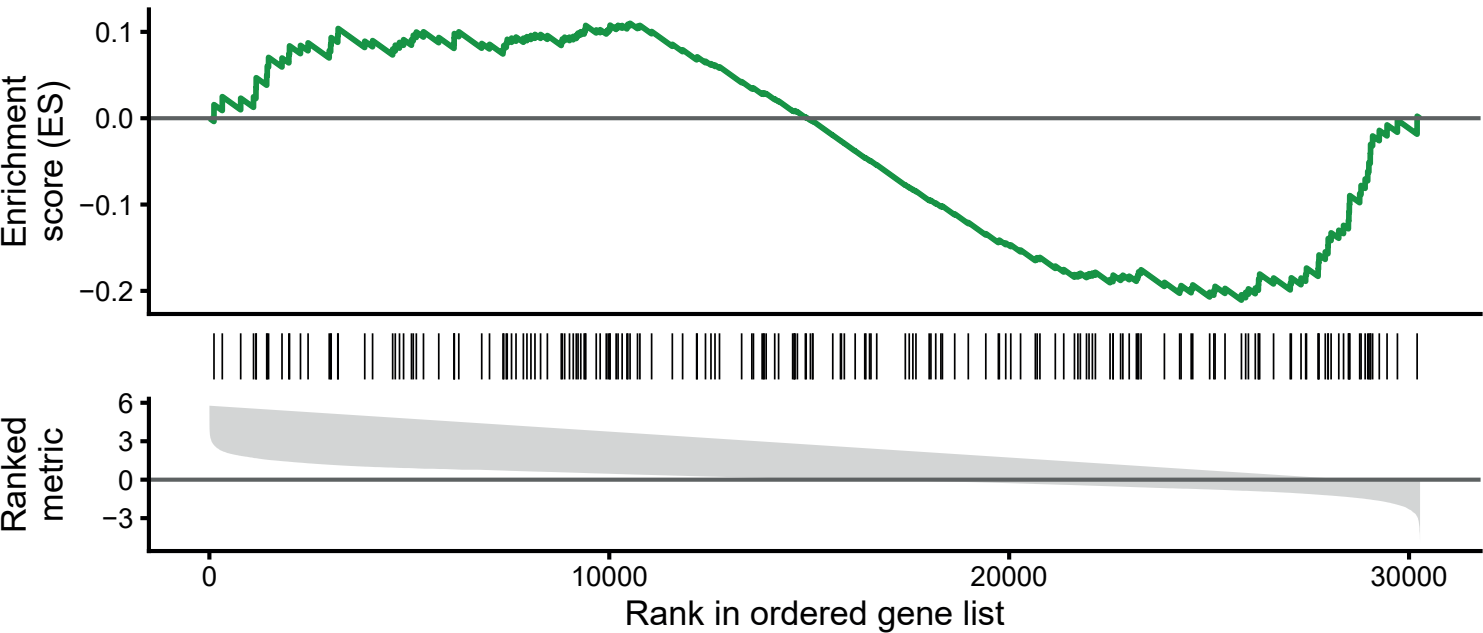


Fig. S3 Additional analysis single nuclei transcriptomic analysis



**P53 pathways enrichment at endpoint, compaired to baseline (paired), in D+Q group**

NES = -1.041 | p = 0.333 | p.adj = 0.626 | Gene set size = 197 | Leading edge = 38 genes



**KEGG\_HEPATOCELLULAR\_CARCINOMA pathways enrichment in D+Q group**

NES = -1.192 | p = 0.105 | Gene set size = 163 | Leading edge = 35 genes

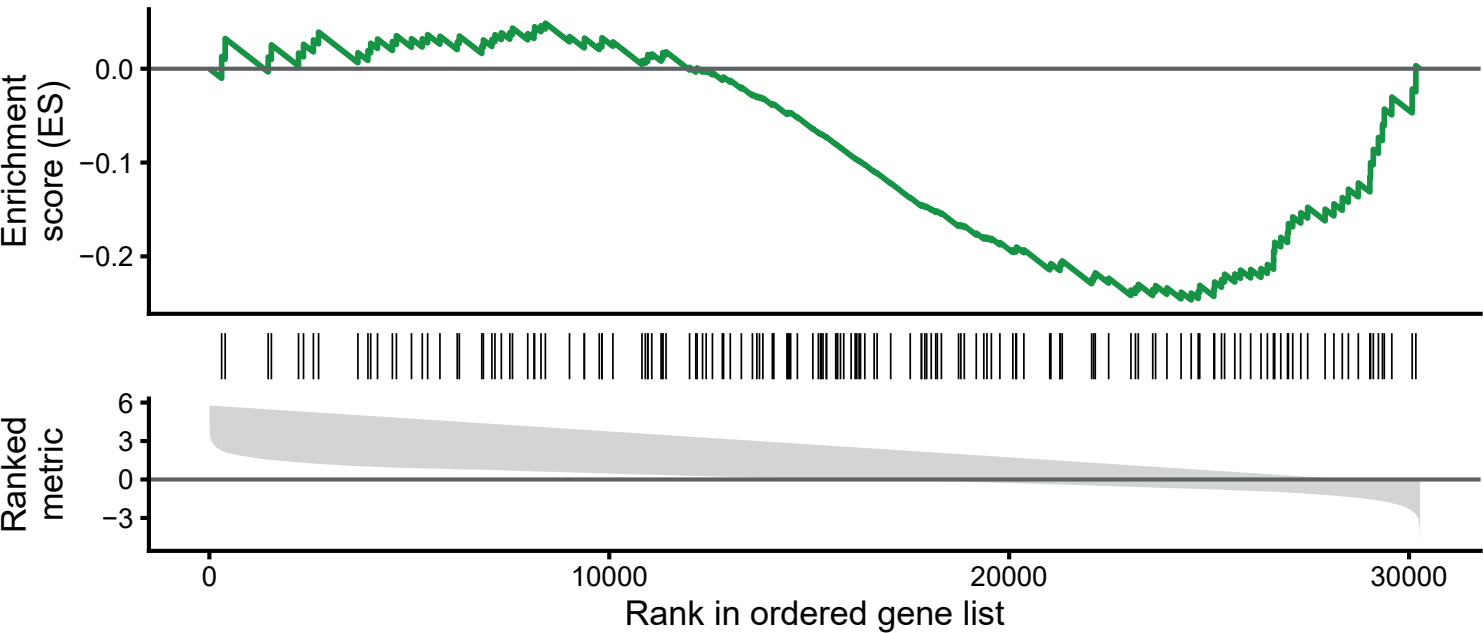
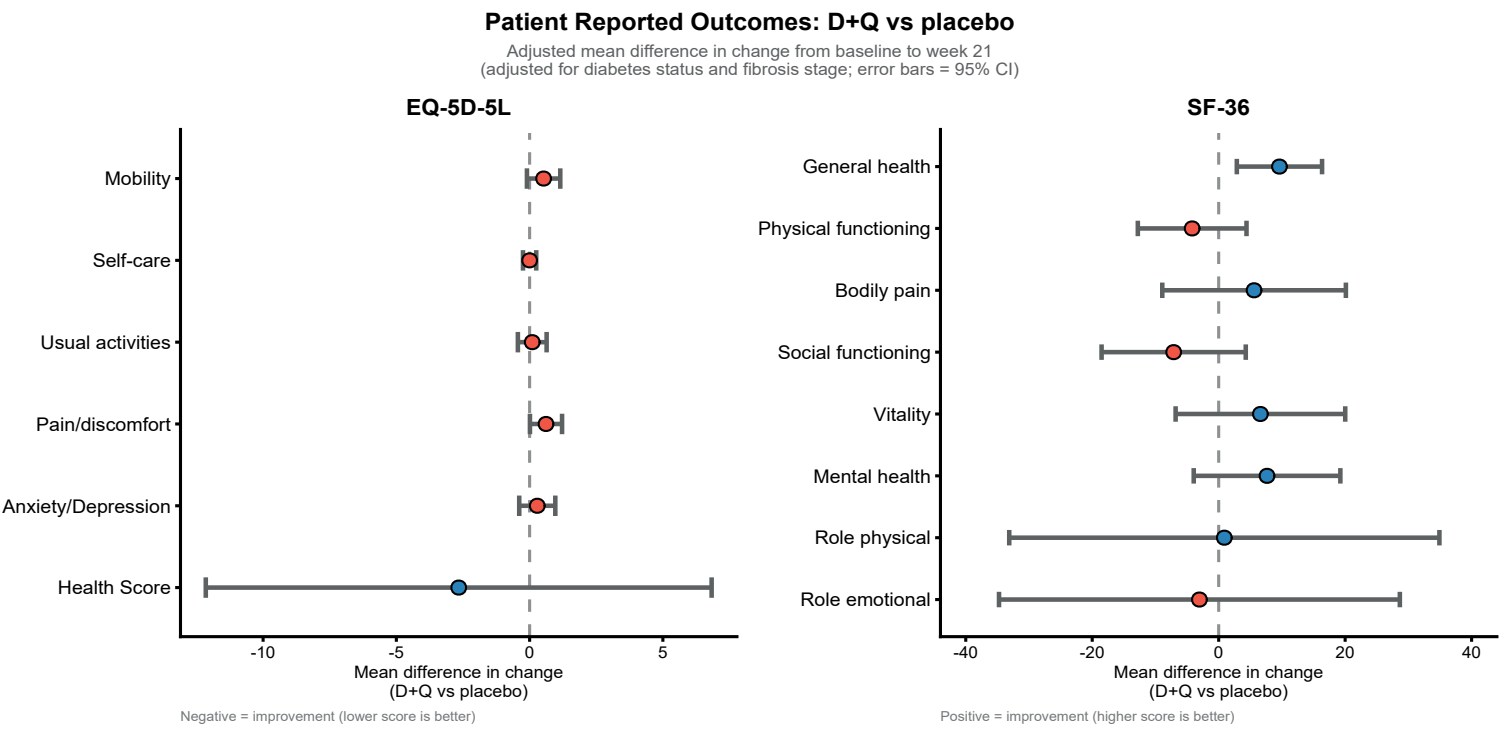


Fig. S5. Patient reported outcomes



## **Supplementary information legends**

### **Suppl. Fig. 1**

*Overview of study design and study duration.*

### **Suppl. Fig. 2**

*A. Heatmap with marker gene expression across cell types. Red indicates genes that are upregulated and blue indicates those that are down regulated. Gene expression is shown as z-scores normalized per gene across clusters*

### **Suppl. Fig.3**

*A. Leading edge of SenMayo enrichment in placebo group at baseline and end of study, with genes determined with fgsea test. Red indicates enrichment and blue indicates a downregulation. Z-score was calculated individually by each sample and then averaged per group. B. Enrichment plot showing SenMayo enrichment after the treatment in the placebo group based on the t-statistics of the pre-ranked genes. Fgsea test P-value estimation is based on an adaptive multi-level split Monte-Carlo scheme. C. Leading edge of SHGS enrichment at baseline and end of study in the D+Q group, with genes determined with fgsea test. Red indicates enrichment of genes and blue indicates down regulation of genes. Z-score was calculated individually by each sample and then averaged per group. D. Enrich plot showing SHGS enrichment after the treatment in the placebo group based on the t-statistics of the pre-ranked genes. Fgsea test P-value estimation is based on an adaptive multi-level split Monte-Carlo scheme E. Enrichment of fibrosis markers per function in the D+Q group at baseline and end of study. Red indicates enrichment and blue indicates downregulation. Z-score was calculated individually by each sample and then averaged per group. F. Enrich plot visualizing fibrosis gene set in the placebo group after treatment based on the t-statistics of the pre-ranked genes. Fgsea test P-value estimation is based on an adaptive multi-level split Monte-Carlo scheme G. Proportional abundance per cell type before and after treatment in the D+Q group. H. Proportional abundance per cell type before and after treatment in the placebo group*

### **Suppl. fig 4.**

*A. Enrich plot visualizing enrichment of p53 pathway after treatment in the D+Q group. B. Enrich plot visualizing KEGG Hepatocellular carcinoma pathway after treatment in the D+Q group.*

### **Suppl. Fig. 5**

*Forest plots show the adjusted mean difference in change from baseline to week21 for D+Q versus placebo. Left panel; for EQ-5D-5L dimensions (mobility, self-care, usual activities, pain/discomfort, anxiety/depression), a negative value indicates improvement; for the health score, a positive value indicates improvement. Right panel: SF-36 domain scores (0–100 scale), where a positive value indicates improvement (higher score = better health). Error bars represent 95% confidence intervals. Blue points indicate improvement in the D+Q group; red points indicate worsening or no change.*

### **Supplementary Table 1**

Concomitant medication use per group during the trial. Data show the number (%) of participants using each medication class. Comparisons between groups were analysed with two-sided Fisher's exact test.

### **Supplementary Table 2**

Supplementary table with cell-type proportional abundance change per group. Mixed-effects logistic regression which models cell-level data with patient as a random effect for proportional abundance change after treatment per group

### **Supplementary Table 3**

Enrichment analysis for cancer related pathways in the D+Q group at 21 weeks compared to baseline.

### **Supplementary Table 4**

Sensitivity analysis for the primary outcome.

### **Supplementary Table 5**

Model assumptions for all ANCOVA analyses with sensitivity analysis where appropriate.

### **Supplementary Table 6**

Sensitivity analysis for ANCOVA analysis with patient reported outcomes.



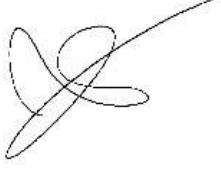
## Dasatinib and quercetin, a combination of senolytics to treat fibrotic non-alcoholic fatty liver disease

|                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
|----------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Protocol ID</b>               | TRUTH                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
| <b>Short title</b>               | The TRUTH study                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| <b>EudraCT number</b>            | 2021-005859-37                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| <b>Version</b>                   | 3. 3                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| <b>Date</b>                      | 10-6-2024                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| <b>Coordinating investigator</b> | Prof. dr. M. Nieuwdorp, internist, Amsterdam UMC – AMC<br>Email: m.nieuwdorp@amsterdamumc.nl<br>Tel: +31 20 5665737                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| <b>Principal investigator(s)</b> | <p>Prof. dr. M. Nieuwdorp, internist Amsterdam UMC – AMC<br/>Email: m.nieuwdorp@amsterdamumc.nl</p> <p>Dr. V.E. Gerdes, internist Amsterdam UMC – AMC<br/>Email: v.e.gerdes@amsterdamumc.nl</p> <p>Dr. N.M.J. Hanssen, internist Amsterdam UMC-AMC<br/>Email: <a href="mailto:n.m.j.hanssen@amsterdamumc.nl">n.m.j.hanssen@amsterdamumc.nl</a></p> <p>Prof. dr. J. Verheij, patholoog Amsterdam UMC.<br/>Email: j.verheij@amsterdamumc.nl</p> <p>Prof. Dr. Ulrich Beuers, MDL-arts, Amsterdam UMC - AMC<br/>Email: u.h.beuers@amsterdamumc.nl</p> <p>Dr. H. Herrema, Amsterdam UMC - AMC<br/>Email: h.j.herrema@amsterdamumc.nl</p> <p>Drs. J. Willemse, Director NLV,<br/>Email: j.willemse@leverpatientenvereniging.nl</p> <p>Drs. A.S. Meijnikman, MD PhD student, Amsterdam UMC - AMC<br/>Email: a.s.meijnikman@amsterdamumc.nl</p> <p>Drs. M. Koning, MD PhD student<br/>Amsterdam UMC-AMC</p> |

Protocol TRUTH versie 3.3 d.d. 10-6-2024 M. Koning, afd. vasculaire geneeskunde Amsterdam1 UMC, locatie AMC

|                               |                                                                                                                                                                                                                                                                             |
|-------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                               | Email: mijra.koning@amsterdamumc.nl                                                                                                                                                                                                                                         |
|                               |                                                                                                                                                                                                                                                                             |
| <b>Sponsor</b>                | Prof. dr. J.M. Prins                                                                                                                                                                                                                                                        |
| <b>Subsidising party</b>      | ZONMW                                                                                                                                                                                                                                                                       |
| <b>Independent expert (s)</b> | Dr D.M. Cohn, internist Amsterdam UMC - AMC<br>Email: d.m.cohn@amsterdamumc.nl<br>Tel: 020 5669111                                                                                                                                                                          |
| <b>Laboratory sites</b>       | Laboratory of Experimental Vascular Medicine,<br>Amsterdam UMC - AMC<br>Dr. J. Kroon<br>Email: j.kroon@amsterdamumc.nl<br><br>Laboratorium algemene klinische chemie<br>(LAKC) Amsterdam UMC – AMC<br>Mirjam Dijkstra, Trial coördinator<br>trialcoordinatorlakc@amc.uva.nl |
| <b>Pharmacy</b>               | Clinical trial Pharmacy Amsterdam UMC – AMC<br><br>trial.apotheek@amsterdamumc.nl                                                                                                                                                                                           |

## PROTOCOL SIGNATURE SHEET

| Name                                  | Signature                                                                          | Date      |
|---------------------------------------|------------------------------------------------------------------------------------|-----------|
| Sponsor: Jan prins                    |  | 10-6-2024 |
| Principal Investigator: Max Nieuwdorp |  | 10-6-2024 |

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## SUMMARY

### Rationale:

Non-Alcoholic Fatty Liver Disease (NAFLD) is estimated to affect approximately 25–30% of the population in Western countries and is now the leading cause of chronic liver disease globally<sup>1</sup>. NAFLD is a progressive liver disease and approximately 30% of individuals progress from simple steatosis to Non-Alcoholic Steatohepatitis (NASH), which can further progress to cirrhosis and hepatocellular carcinoma. In the Netherlands, it is estimated that 2.5 million people have NAFLD. This number is thought to increase by 50% in the next 10 years. This is mainly due the rising prevalence of obesity and type 2 diabetes, and an aging population, which are main drivers of NAFLD<sup>2</sup>. Independent of other cardiometabolic diseases, cardiovascular disease is the leading cause of death in individuals with NAFLD, followed by extrahepatic malignancies and liver-related complications. NAFLD results in sustained healthcare costs and economic losses, and reduced health-related quality of life.

It is now widely accepted that liver fibrosis is a result of liver injury secondary to NAFLD and is a major predictor for liver-related and overall mortality in individuals with NAFLD<sup>3–5</sup>. As of to date, no treatment is available that proved to be successful to target hepatic fibrosis. The only therapeutic options currently available therefore are diet and lifestyle changes and control of concomitant metabolic diseases<sup>6</sup>. Unfortunately, this inevitably leads to polypharmacy, which decreases treatment adherence and increases the risk of adverse events and interactions with other drugs.

Recently, cellular senescence has been put forward as a causal factor in the development and progression of NAFLD and NAFLD-related liver fibrosis<sup>7–9</sup>. Cellular senescence is one of the hallmarks of aging and is defined as a stable arrest of the cell cycle coupled to specific phenotypic changes<sup>3–5</sup>. Senescent cells secrete a collection of proteins called the senescence-associated secretory phenotype (SASP). This pro-inflammatory secretome drives age-related tissue dysfunction. Interestingly, metabolic dysregulation is thought to favor cellular senescence in several tissues involved in the pathogenesis of NAFLD such as the liver, pancreas, and adipose tissue, further perpetuating metabolic dysregulation<sup>10,11</sup>. Of interest, cellular senescence can be targeted using senolytics. The combination of dasatinib, which is an EMA-approved tyrosine kinase inhibitor, and the antioxidant quercetin, which is a flavonol present in many fruits and vegetables, successfully clears senescent cells. Recent work in humans and rodents have shown that tissue function, including liver metabolism, can be recovered by clearing senescent cells with senolytics<sup>10,12</sup>.

Considering the role of senescence in NAFLD-related fibrosis, dasatinib plus quercetin might be an interesting therapeutic intervention to meet the high unmet clinical need for NAFLD. Importantly, dasatinib and quercetin have a strong long-term safety profile<sup>13</sup>. We

therefore aim to perform a double-blind randomized controlled proof-of-principle study in which patients with NAFLD-related liver fibrosis will be treated with dasatinib plus quercetin. The treatment will be intermittent (three days per week for three weeks) followed by a four-week medication-free period. This treatment cycle will be repeated three times.

**Objective:** To examine the effect of oral dasatinib plus quercetin on liver fibrosis as assessed by histology in individuals with biopsy-proven NAFLD with fibrosis by performing a double-blind randomized controlled proof-of-principle study. The primary endpoint is the binary outcome improvement of fibrosis with at least 1-point without worsening of fibrosis and NAFLD score based on histology after 21 weeks (yes/no).

**Study design:** Double-blind randomized controlled proof-of-principle study in which 30 patients with fibrotic NAFLD will use dasatinib plus quercetin intermittently three days per week for three weeks, followed by a four-week medication-free period. This treatment cycle will be repeated three times and thus a total of 21 weeks.

**Study population:** The study population will comprise adult individuals  $\geq 18$  years, with biopsy-proven NAFLD with liver fibrosis (Steatosis Activity and Fibrosis score  $\geq 2$  based on histology) but no cirrhosis.

**Intervention:** The intervention group will intermittently receive orally administered dasatinib (100 mg/day) plus quercetin (1000 mg/day) on three consecutive days for three consecutive weeks followed by a four-week medication free period. This cycle will be repeated three times. The placebo group will receive matched placebo capsules according to the same treatment schedule.

**Main study parameters/endpoints:** The primary endpoint is the binary outcome improvement of fibrosis with at least 1-point without worsening of fibrosis and/or NAFLD/NASH scores (i.e., steatosis, lobular inflammation, hepatocyte ballooning) based on histology after 21 weeks (yes/no). Individuals will be labeled as responder or non-responder.

In addition, the following secondary objectives will also be investigated: changes in liver-related histological parameters and high dimensional RNAseq data; changes in biomarkers of NAFLD including liver function tests; changes in glucose metabolism related parameters; Changes in fecal microbiota composition and bileacid composition; changes in patient reported outcomes; safety and tolerability; changes in blood pressure, blood pressure and ECG. Also online dietary data ([mijn.voedingscentrum.nl/nl/eetmeter](https://mijn.voedingscentrum.nl/nl/eetmeter)) and freestyle libre data are taken at beginning and end of the trial.

**Nature and extent of the burden and risks associated with participation, benefit and group relatedness:**

At the Amsterdam UMC a NAFLD/NASH clinic is established, giving us the opportunity to build a large cohort including >500 individuals with ranging liver disease and fibrosis. We Protocol TRUTH versie 3.3 d.d. 10-6-2024 M. Koning, afd. vasculaire geneeskunde Amsterdam<sup>8</sup> UMC, locatie AMC

recently published a RCT using repetitive liver biopsies<sup>14</sup>, which was performed even before the NAFLD clinic and cohort was established, with a similar number of individuals, underscoring feasibility. With the help of the Nederlandse Leverpatienten Vereniging (NLV, director drs J. Willemse), we estimated that the total study burden is in balance with potential risks and benefits of participation for patients with liver disease in this study. Nevertheless, the study can be considered extensive and invasive for the participants due to following procedures:

Liver biopsy: Study participants will undergo a liver biopsy at the end of the study (or at baseline when no baseline biopsy was performed within six months prior to the start) because a Fibroscan, MRI or ultrasound, although non-invasive, are inferior for the accurate diagnosis and are therefore not used in clinical trials for primary endpoints<sup>15</sup>. Although a liver biopsy is an invasive procedure, it remains the gold standard for the diagnosis of NAFLD and is therefore required for the interpretation of the results<sup>15</sup>.

Given the fact that the individuals are recruited via our outpatient NAFLD clinic, oftentimes a biopsy of the liver has already been performed. Therefore, only one additional biopsy is necessary for this trial. Liver biopsies are performed after informed consent, via the AMC intervention radiology department or gastroenterology department for an ultrasound-guided liver biopsy to assess NAFLD/NASH histology. As an experienced hepatologist or interventional radiologist will perform the liver biopsy, the risk of complications will be very low (<0.1%) and comprises mostly bleeding at the biopsy sites (see METC 2017\_311, 2019\_61 and 2022\_236). Bleeding disorders therefore are an exclusion criterion.

Safety and tolerability: A possible pitfall is the safety and tolerability of the senolytic drug. However, intermittent dasatinib plus quercetin treatment has an exceptionally good safety profile. Worldwide, hundreds of individuals are enrolled in clinical trials using dasatinib plus quercetin (Table 2, page 25-26) for a variety of conditions, with no severe or serious side effects so far. Dasatinib alone has been approved as prescription drug in the EU for various cancers and for fibrotic diseases (scleroderma, progressive systemic sclerosis), usually at doses of 100 mg/day for life. Of interest, in contrast to other tyrosine kinase inhibitors, dasatinib usage is not associated with an increased risk of hepatotoxicity<sup>16</sup>. Recently, the suggestion that dasatinib is associated with improved glucose metabolism and decreased need for glucose lowering medication was further substantiated<sup>17</sup>. Side-effects of dasatinib include abdominal pain, vomiting, diarrhea, musculoskeletal pain, tiredness, and myelosuppression. Another side effect, which may occur after months of continuous use, include peripheral edema and pleural effusions. Importantly, it has been described that a twice daily dasatinib regimen, as well as a history of cardiac or autoimmune disease, are suspected as predisposing factors<sup>18,19</sup>. To minimize such risks, these predisposing factors are exclusion criteria in the present RCT. In addition, pleural effusion is driven by a reactive oxygen species (ROS) dependent mechanism. Importantly, these side effects are reversible. In addition, a recent study showed that the increased endothelial permeability, which drives the side-effects, can be counteracted using antioxidants such as quercetin<sup>20</sup>. Hence, quercetin is not only a senolytic agent by itself, but the combination of dasatinib plus quercetin appears a highly effective and safe drug.

combination to selectively target age-related diseases via an intermittent treatment strategy<sup>13</sup>.

Treatment adherence: In the past decades, extensive research has given important insight into the many complex factors that determine how and why individuals take prescribed drugs. The focus has been on the consumers factors that relate to non-compliance. However, there is also growing awareness that factors beyond consumers' control can affect adherence. It is now widely accepted that communication in its entirety is critical, and it is increasingly accepted that interventions to improve adherence should focus more broadly on patient context and healthcare system. Patient-centered care and shared decision-making, together with increased attention to barriers that may be targets for interventions is pivotal.

With the input of the NLV (Dutch Liver Patient Society), we created a fundamental basis to make our intermittent treatment strategy attractive and to increase compliance similar to once weekly GLP-1 injections or vitamin D supplementation treatment strategy<sup>21</sup>. In this regard, the NLV was closely involved in the currently acquired ZONMW GGG grant (number 10140262110036) that provided funding to execute this TRUTH trial. The NLV mentioned that regular contact with patients should be implemented in the study to remind the individuals to take their medication to increase adherence. If an individual is found to be non-compliant, the investigator will remind the individual of the importance of following the treatment instructions. Treatment compliance of the trial products will be assessed by asking individuals about missed doses and monitoring of dosing diaries. Information about compliance and missed doses will be described in the individual's medical record.

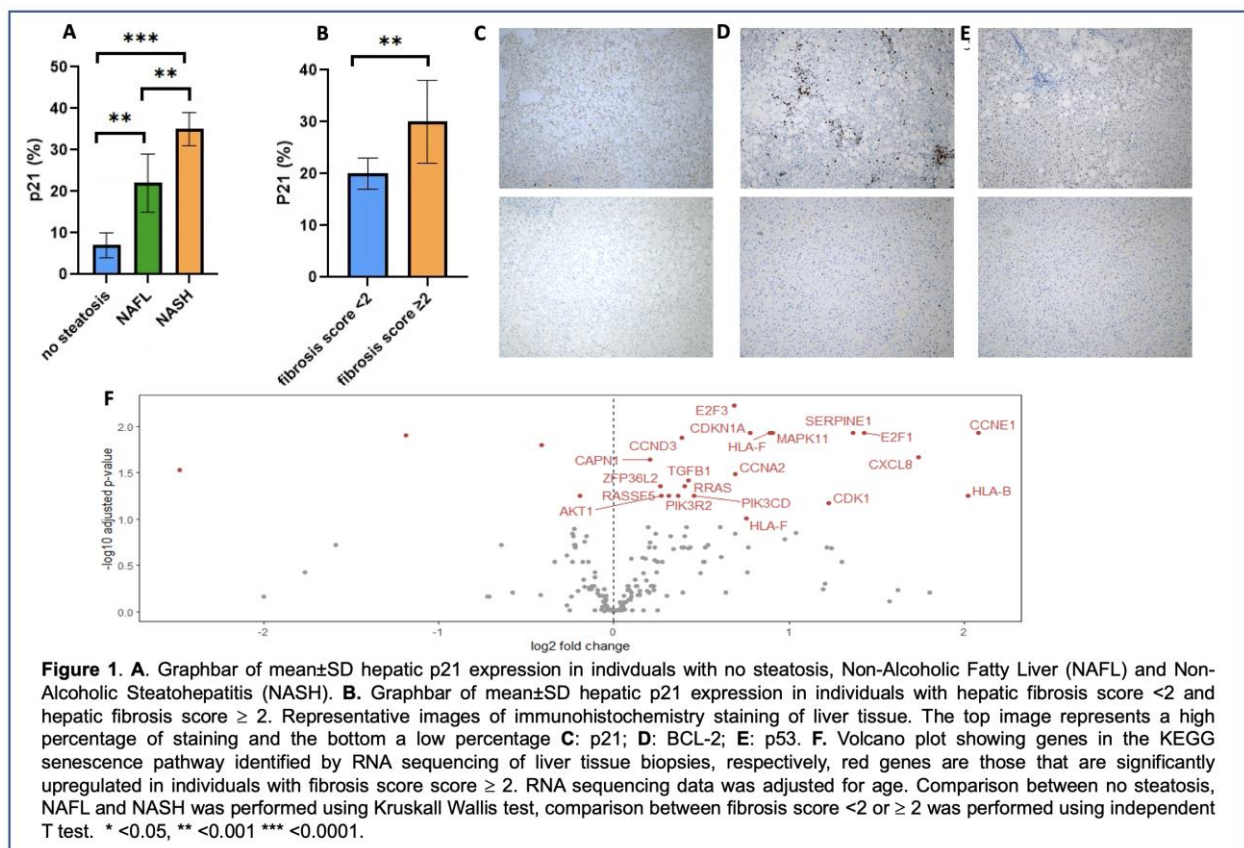
All in all, the proposed trial is important considering the fact that there is currently no registered treatment for NAFLD and more importantly for related liver fibrosis. There is no doubt that with advancing liver fibrosis, there is significant morbidity and mortality. This was recently underscored by a meta-analysis showing that the relative risk for all cause mortality 3.42 (95% CI, 2.63-4.46); liver-related mortality RR, 11.13 (95% CI, 4.15-29.84); liver transplant RR, 5.42 (95% CI, 1.05-27.89); and liver-related events RR, 12.78 (95% CI, 6.85-23.85) increased with increasing stage of fibrosis. Moreover, patients with NAFLD/NASH often have cardiometabolic comorbidity, which can lead to polypharmacy with associated health risks and non-compliance. Senolytics can be orally administered intermittently, which reduces the risks of adverse effects and drug interactions. In view of the expired patent of dasatinib, targeting senescence might be a safe and innovative way to use generic and safe medication to reverse progressive stages of NAFLD/NASH.

## 1. INTRODUCTION AND RATIONALE

Non-alcoholic fatty liver disease (NAFLD) is one of the major global non-communicable diseases affecting 25–30% of the population in Western countries. It is one of the leading indications for liver transplantation and has shown a 7-fold increase between 2002 and 2016 according to the European Liver Transplant Registry. NAFLD represents a spectrum of liver diseases, with clinical and histological abnormalities ranging from simple steatosis (NAFL) without significant fibrosis to steatohepatitis (NASH), with varying stages of fibrosis. The transition from NAFL to NASH identifies a more aggressive disease phenotype with increased likelihood of developing complications such as cirrhosis and hepatocellular carcinoma (HCC). Approximately 7–30% of those with NAFLD fulfill the criteria for NASH. The risk of developing HCC from NASH is estimated to be 5.29 per 1000 people (95%CI: 0.75–37.56) and 10.6 per 1000 people years from NASH cirrhosis. There is now substantive and consistent evidence that NAFLD-related fibrosis is the strongest predictor of future all-cause mortality and morbidity, with a 5–12-fold increase in Relative Risk of death and liver-related events, including liver failure, transplantation, and liver cancer. NAFLD results in sustained healthcare costs and economic losses, and reduced health-related quality of life. Targeting NAFLD and especially NAFLD-related fibrosis with the aim to prevent disease progression is an incremental component of patient management strategies. However, most of the recent phase 3 trials testing novel compounds have been prematurely terminated and as of now there is still no specific treatment approved for NAFLD<sup>6</sup>. In the past decades, cellular senescence has generated a lot of interest among researchers because of its involvement in both the normal aging process and common human diseases<sup>22,23</sup>. During senescence, cells undergo alterations that include telomere shortening, nuclear area enlargement, and genomic and mitochondrial DNA damage, leading to irreversible cell cycle arrest, and secretion of proinflammatory cytokines called the senescence-associated secretory phenotype (SASP)<sup>10</sup>. Senescent cells do not undergo apoptosis. Instead, they have upregulated pro-survival pathways called senescent cell anti-apoptotic pathways (SCAPs) while key apoptotic mediators are downregulated<sup>24,25</sup>. Evidence suggests that the complex process of senescence is involved in the development of a plethora of chronic diseases including metabolic and inflammatory disorders. Recently, several human and animal studies have emphasized the involvement of senescence in the pathogenesis and development of liver steatosis including the progression of the disease as characterized by the additional emergence of inflammation, hepatocyte ballooning, and liver fibrosis<sup>10,26,27</sup>. Indeed, hepatocytic senescence has been shown to impair mitochondrial  $\beta$ -oxidation, thereby hindering fatty acid elimination, and promoting triglyceride accumulation<sup>28</sup>. Moreover, in a prospective trial, hepatocytic senescence proved to be an independent predictor of disease progression and liver fibrosis<sup>27,29</sup>. Concomitantly, SASP components are abundantly present in adipose tissues of individuals with obesity and promote insulin resistance and inflammation, which are heavily involved in NAFLD<sup>30</sup>.

To further substantiate the association between NAFLD and senescence in humans, we analysed baseline data from the ongoing BARIA study<sup>31</sup>. In this study, both clinical data, liver histology as well as high-dimensional data including transcriptomics data of the liver are collected from individuals that are scheduled for bariatric surgery. We first addressed

cellular senescence in liver biopsies from 63 matched individuals with no steatosis, NAFL or NASH. To this end, the well-known and robust biomarkers p21 (cyclin-dependent kinase inhibitor 1A), apoptosis inhibitory protein BCL-2 and p53 were quantified using immunohistochemistry<sup>32</sup>. In line with previous reports, expression of senescence markers were positively associated with NAFLD scores (Figure 1). Also, a significant difference was observed between individuals with and without a fibrosis score  $\geq 2$ . In addition, when dividing individuals into two groups according to the fibrosis score  $\geq 2$ , the liver transcriptome revealed that genes associated with senescence were increased in individuals in the latter group. These results further underscore that senescent cells are increased with NAFLD progression and hepatic fibrosis. To what extent hepatocyte cellular senescence is a mere consequence of the metabolic dysregulation and inflammatory phenomena in NAFLD or a causal factor remains to be investigated.



Already in 1995, it was discovered that senescent cells are resistant to apoptosis<sup>33</sup>. It took another decade to reveal that senescent cells uniquely depend on SCAPs, which permit senescent cells to survive their own SASP<sup>25</sup>. Hence, SCAPs were identified as the Achilles heel of senescent cells<sup>25</sup>. Due to tremendous research efforts of the field, it is now possible to successfully target senescent cells. Multiple senolytic candidates have been discovered and are among currently available drugs to target senescent cells in a multitude of diseases. Dasatinib, which is an EMA-approved oral tyrosine kinase inhibitor and the antioxidant quercetin, which is a flavonoid; present in many fruits and vegetables,

Protocol TRUTH versie 3.3 d.d. 10-6-2024 M. Koning, afd. vasculaire geneeskunde Amsterdam UMC, locatie AMC

successfully induce apoptosis in senescent cells in rodent and human studies<sup>33</sup>. Dasatinib by itself, has an antidiabetic effect and could possibly be considered as a new anti-diabetic therapy<sup>67</sup>. This is in line with previous studies suggesting that senescence also plays a role in insulin resistance and the development of diabetes type 2<sup>70</sup>. Studies in mice showed that senescence markers p53 and p16 are associated with insulin resistance and treatment with dasatinib plus quercetin improved insulin resistance<sup>68-69</sup>. Of interest, Quercetin is known for altering the gut microbiome<sup>65</sup>. It does so by reducing microbiome imbalance and diminution of endotoxemia-mediated TLR-4 pathway induction. This results in less inflammation, cellular stress and blockage of lipid metabolism gene expression deregulation<sup>65</sup>. We recently showed a causal relation between the gut microbiome and NAFLD<sup>64</sup>. Vice versa the gut microbiome influences the beneficial effect of quercetin on atherosclerosis underscoring the pleiotropic effects of these medications<sup>66</sup>.

The combination of dasatinib and quercetin acts on multiple SCAP network nodes and thereby increases the potency towards clearing senescent cells<sup>25</sup>. The combination of dasatinib plus quercetin strongly reduces off-target apoptotic effects on non-senescent cell types, such as platelets and immune cells. This prevents 'panolytic' actions and thereby flattens the side-effect profile. Moreover, according to *in vitro* data and later data from clinical trials, a brief disruption of pro-survival pathways is adequate to clear senescent cells<sup>8,9</sup>. Because senescent cells take weeks to reaccumulate, senolytics can be administrated intermittently, which reduces the risk of adverse effects compared to continuous treatment.

Translating these exciting results to the clinic, dasatinib plus quercetin as the first-in-human senolytic therapy showed efficacy in treating idiopathic pulmonary fibrosis, a fatal disease associated with the appearance of senescent cells in the lungs<sup>34,35</sup>. Additionally, the same combination was used as treatment of obesity/diabetic kidney dysfunction and resulted in the decrease of adipose tissue crown-like structures, which are areas of fibrosis<sup>36</sup>. Proof of concept pilot trials using senolytics have shown that they clear senescent cells, reduce inflammation, alleviate frailty, and improve clinical outcome in humans. Clinical trials for diabetes, idiopathic pulmonary fibrosis, Alzheimer's disease, COVID-19, osteoarthritis, osteoporosis, eye diseases and bone marrow transplant and childhood cancer survivors are underway, using dasatinib plus quercetin or other senolytic drugs (Table 2, page 25-26). The striking translational results (i.e., *in vitro*, rodents, humans), the strong safety profile of dasatinib plus quercetin and the fact that there is currently no pharmacological treatment for NAFLD-related liver fibrosis justifies a trial using dasatinib plus quercetin for the treatment of NAFLD-related fibrosis.

In collaboration with the NLV, we aim to perform a double-blind randomized controlled proof-of-principle study in which individuals with NAFLD-related fibrosis will be treated with dasatinib plus quercetin intermittently three days per week for three weeks, followed by a four-week medication-free period. This treatment cycle will be repeated three times.

## 2. OBJECTIVES

### 2.1 Primary Objective:

The primary objective is to examine the effect of dasatinib plus quercetin on liver fibrosis as histologically assessed in individuals with biopsy-proven NAFLD with fibrosis by performing a double-blind randomized controlled proof-of-principle study. The primary endpoint is the binary outcome improvement of fibrosis with at least 1-point without worsening of fibrosis and NAFLD score based on histology after 21 weeks (yes/no).

### 2.2 Secondary Objective(s):

1. To examine the effect of dasatinib plus quercetin on liver related histological parameters and high dimensional data by addressing the:
  - mean number of senescent cells baseline vs end of treatment (week 21).
  - percentage of patients with reversal of NAFLD and no worsening of fibrosis (week 21).
  - differences in hepatic gene expression (determined by RNA seq) baseline vs end of treatment (week 21).
  - differences in NAFLD activity score (NAS) baseline to end of treatment (week 21).
  - differences in activity component of NAFLD using the steatosis-activity-fibrosis (SAF) score baseline vs end of treatment (week 21).
  - Differences in EPOS 7-tier staging system score baseline to end of treatment (week 21)
2. To examine the effect of dasatinib plus quercetin on biomarkers of NAFLD by addressing the:
  - Fibrosis-4 score (Fib-4 score) and NAFLD Fibrosis Score (NFS) baseline vs end of treatment (week 21).
  - circulating liver enzymes and synthesis function (e.g., ALT, AST, and  $\gamma$ GT, albumin, INR) baseline vs end of treatment (week 21).
  - liver stiffness and liver steatosis (with controlled attenuation parameter) using Fibroscan baseline vs end of treatment (week 21).
3. To examine the effect of dasatinib plus quercetin on glucose metabolism-related parameters by addressing the:
  - glycosylated haemoglobin type A1c (HbA1c), fasting plasma glucose (FPG), fasting insulin and glucagon baseline vs end of treatment (week 21).  
Homeostatic model assessment of insulin resistance (HOMA-IR) baseline vs end of treatment (week 21). Glucose variability (determined by 2 weeks of Freestyle libre at begin and end of the trial).

4. To examine the effect of dasatinib plus quercetin on patient reported outcome including by addressing:
  - RAND-36 and EQ-5D-5L: physical and mental component summary scores and scores on the individual sub-domains: Physical functioning, daily functioning, bodily pain, general health, vitality, social functioning, and role emotional and mental health, baseline vs end of treatment (week 21).
5. To examine the safety of dasatinib plus quercetin as assessed by:
  - Number of treatment-emergent adverse events during the trial, during the trial.
  - Number of treatment-emergent myelosuppression, during the trial.
  - Number of treatment emergent infections, during the trial.
  - Number of patients discontinuing treatment due to gastrointestinal adverse events, during the trial.
6. To examine the effect of dasatinib plus quercetin on:
  - Pulse, blood pressure, blood pressure and ECG, baseline vs end of treatment (week 21).
  - Physical examination, baseline vs end of treatment (week 21).
  - Haematology (haemoglobin, thrombocytes, erythrocytes, leucocytes, differential count), during the trial.
  - Biochemistry (creatinine, creatinine phosphokinase, urea, bilirubin (total), alkaline phosphatase, ferritin, sodium, potassium, calcium), during the trial
  - Fecal microbiota composition (morning stool and bile acids)

### 3. STUDY DESIGN

This study is designed as a single center double-blind randomized controlled proof-of-principle study in 30 patients with NAFLD-related liver fibrosis and will be performed in the Amsterdam UMC. A placebo arm is highly recommended in NAFLD, and NAFLD related trials by an expert panel because some individuals will respond to standard care (i.e., lifestyle, diet, and treatment of co-morbidities) with percentages up to 10%<sup>15</sup>.

After inclusion, the patients will be randomized in the treatment or placebo arm of the study. Each treatment arm will contain 15 patients with biopsy-proven NAFLD with a fibrosis score  $\geq 2$ . Patients randomized into the treatment arm will use dasatinib 100 mg plus quercetin 1000 mg intermittently three days per week for three weeks, followed by a four-week medication free period. This cycle will be repeated three times. Patients randomized into the placebo arm will receive matched number of placebo capsules according to this schedule. The total follow-up is 21 weeks. Five study visits are planned during the entire course of the study including at screening, at baseline (start of the treatment) and then at seven, 14, and 21 weeks after the start of the treatment. Dosing and intermittent treatment strategy are based on strong pre-clinical data and on the recently published proof of principle studies in obesity/diabetic kidney disease and idiopathic pulmonary fibrosis - diseases characterized by accumulation of senescent cells.

NAFLD-related liver fibrosis status will be examined by an expert panel in transcutaneous liver biopsies at baseline and 21 weeks. The histology sections are blinded for the expert panel because the patient number cannot be linked to the randomization arm. Non-invasive liver transient elastography and blood sampling including parameters for glucose metabolism and biomarkers for NAFLD will be performed during each study visit. In addition, before and after each treatment cycle, complete blood counts will be performed to monitor myelosuppression. Before and after each treatment cycle, pulse and blood pressure will be measured. In addition, before and after each treatment cycle, an ECG will be performed to monitor risk of arrhythmias.

## 4. STUDY POPULATION

### 4.1 Population

This study is performed in individuals with NAFLD-related liver fibrosis and will be performed in the Amsterdam UMC in collaboration with the NLV. NAFLD-related fibrosis must be diagnosed with the gold standard, a liver biopsy. Individuals who were recently diagnosed with NAFLD-related fibrosis and had a fibrosis score  $\geq 2$  (i.e., perisinusoidal and portal/periportal fibrosis) based on a liver biopsy that was performed in the past six months are eligible for inclusion. No additional liver biopsy must be performed. Our dedicated NAFLD outpatient clinic, located at two sites in Amsterdam, allows a rapid start with inclusion of patients with NAFLD (biopsy proven). We aim to mostly include individuals that already underwent a liver biopsy

In addition, patients will also be recruited through advertisement. Bearing in mind the invasive nature of a liver biopsy, we designed an inclusion strategy in collaboration with the NLV to dramatically lower the chance of performing a liver biopsy in individuals who do not have NAFLD-related fibrosis. The screening procedure is divided into three parts.

1. Assessment of inclusion and exclusion criteria and measurement of non-invasive test for liver fibrosis using the Fibrosis-4 (FIB-4) index for liver fibrosis.

The (FIB-4) index will be calculated using the formula:  $\text{Age (years)} \times \text{AST U/l} / [\text{PLT (109/l)} \times \text{ALT}^{1/2} (\text{U/l})]$ . The negative predictive value for excluding patients with a fibrosis score  $<2$  is excellent. Those patients with FIB-4  $>1.5$  will undergo a non-invasive liver elastography. Or if they already had a liver elastography score  $>7.5$  kilopascal during outpatient clinic visits.

2. Liver elastography (Fibroscan).

Liver elastography is a type of imaging test that checks the liver for fibrosis and is now considered to be the most promising noninvasive method for assessing liver fibrosis. The technique is fast, reproducible and does not cause discomfort or risks to the patient. The negative and positive predictive value for liver fibrosis is excellent. Patients that have a liver elastography score  $>7.5$  kilopascal will undergo a liver biopsy, which is the last step in the screening procedure.

3. Ultrasound-guided liver biopsy

The liver biopsy is still the gold standard for diagnosing NAFLD and NAFLD-related liver fibrosis. The biopsy will be performed by an experienced intervention radiologist from the AMC Department of Radiology or a hepatologist from the AMC department of gastroenterology as done previously (see METC 2017\_311, 2019\_61 and 2022\_236). The histopathological diagnosis of NAFLD and liver fibrosis will be assessed by an expert panel using the SAF (Steatosis, Activity, Fibrosis) scoring system. If the patient has NAFLD-related fibrosis with a fibrosis score  $\geq 2$ , the patient is eligible for inclusion.

## 4.2 Inclusion criteria

In order to be eligible to participate in this study, individuals must meet all of the following criteria:

- Adult individuals, age  $> 18 \leq 70$  years
- NAFLD according to Non-invasive liver tests (FIB-4, APRI)
- Individuals agree to have a liver biopsy performed after the treatment
- Compensated liver disease with the following hematologic and biochemical criteria on entry into study:
  - o ALAT  $< 10 \times$  ULN
  - o Hemoglobin  $> 11$  g/dL for females and  $12$  g/dL for males
  - o White blood cell (WBC)  $> 2.5$  K/  $\mu$ L
  - o Neutrophil count  $> 1.5$  K  $\mu$ L
  - o Platelets  $> 100$  K/ $\mu$ L
  - o Total bilirubin  $< 35$   $\mu$ mol/L
  - o Albumin  $> 30$  g/L
  - o TP  $> 80\%$  or INR  $< 1.4$
  - o Serum creatinine  $< 1.3$  mg/dL (men) or  $< 1.1$  mg/dL (women) or estimated glomerular filtration rate (eGFR)  $> 60$  mL/min/1.73m<sup>2</sup>
- Have a stable weight defined by no more than a 5% loss of initial body weight
- Negative pregnancy test or post-menopausal. Women with childbearing potential (i.e. fertile, following menarche and until becoming post-menopausal unless permanently sterile) must be using a highly effective method of contraception (i.e. combined (estrogen and progestogen containing) hormonal/ progestogen-only hormonal contraception associated with inhibition of ovulation, intrauterine device, intrauterine hormone-releasing system, bilateral tubal occlusion, vasectomised partner, sexual abstinence)
- Subjects should be able to give informed consent

## 4.3 Exclusion criteria

Individuals who meet any of the following criteria will be excluded from participation in this study:

- Evidence of another form of liver disease
- History of sustained excess alcohol ingestion: daily consumption  $> 30$  g/day (3 drinks per day) for males and  $> 20$  g/day (2 drinks per day) for females
- Unstable metabolic condition: weight change  $> 5$  kg in the last three months, diabetes with unstable glycaemic control introduction of an antidiabetic or anti-obesity drug; malabsorptive or restrictive bariatric (weight loss) surgery in the past 6 months prior to screening
- Bariatric surgery
- ingestion of drugs known to produce hepatic steatosis including corticosteroids, high-dose oestrogens, methotrexate, tetracycline or

- amiodarone in the previous 6 months
- Significant systemic or major illnesses other than liver disease, including congestive heart failure (class C and D of the AHA), unstable coronary artery disease, cerebrovascular disease, pulmonary disease, renal failure, organ transplantation, serious psychiatric disease, active malignancy, compromised immunity
- Body mass index (BMI) >45 kg/m<sup>2</sup>
- Type 1 diabetes
- Haemostasis disorders or current treatment with anticoagulants
- Contra-indication for liver biopsy
- History of/or current cardiac dysrhythmias and/or a history of cardiovascular events including myocardial infarction, except patients with only well-controlled hypertension
- QTc >450 msec on ECG
- Individuals who are lactose hypersensitive or have lactase deficiency
- Pregnancy/lactation or inability to adhere to adequate contraception in women of child-bearing potential.
- Use of prescribed drugs dependent on CYP3A4 with narrow therapeutic window and strong inducers or inhibitors of CYP3A4
- Use of H<sub>2</sub>-antagonists and/or Proton Pump Inhibitors

#### **4.4 Sample size calculation**

Bearing in mind that the primary endpoint is the binary outcome improvement of fibrosis with at least 1-point without worsening of fibrosis and NAFLD score based on histology after 21 weeks (yes/no). The sample size calculation is therefore based on an aim to detect a difference in the probability of achieving an improvement of fibrosis with at least 1-point without worsening of fibrosis and/or NAFLD scores at week 21 between the dasatinib plus quercetin and placebo at 5% significance level, using Pearson chi-square test with normal approximation. The power is set to 80%.

It is expected that some subjects will respond to standard care (i.e., lifestyle, diet and treatment of co-morbidities) and have a decrease in fibrosis, with percentages up to 10%<sup>15</sup>. It is uncertain how many subjects on dasatinib plus quercetin should be expected to achieve an improvement of NAFLD related fibrosis. According to the preliminary report from an early, open-label clinical trial of 3 days, oral dasatinib plus quercetin administration in individuals with diabetes complicated by renal dysfunction, dasatinib plus quercetin decreased adipose tissue inflammation as evident by reduced macrophage number and reduced formation of crown-like structures. Crown like structures are areas of fibrosis. A near complete decrease in adipocyte fibrosis was observed concomitant with an overall decrease of 10-20% in senescence markers in adipose tissue (p16<sup>INK4a</sup>+, p21<sup>+</sup>, and SA-βgal<sup>+</sup> senescent cells). As a conservative assumption, it is anticipated that more than 52% of the individuals treated with dasatinib plus quercetin will achieve an improvement of fibrosis with at least 1 point without worsening of fibrosis or NAFLD scores. Whereas in the placebo arm, 10% of the individuals has an improvement.

Based on the assumptions above, the required number of randomized subjects per arm found to be 15. Hence, 30 individuals with NAFLD related fibrosis are needed in total. In case of withdrawal from of an individual participant, an additional participant will be included. We expect a drop-out of 10%, hence 33 individuals need to be recruited. Of note, all power calculations were performed with an online power calculation ([www.biomath.info/power/](http://www.biomath.info/power/)).

## 5. TREATMENT OF SUBJECTS

### 5.1 Investigational product/treatment

The intervention group will receive intermittent oral dasatinib (100 mg/day) plus quercetin (1000 mg/day) orally on three consecutive days for three consecutive weeks followed by a four-week medication free period. This cycle will be repeated three times.

#### **Dasatinib**

Dasatinib is a tyrosine kinase inhibitor and has been approved as a prescription drug in the EU for various cancers and for fibrotic diseases (scleroderma, progressive systemic sclerosis), usually at doses of 100 mg/day for life. Unlike standard treatment regimens, in this trial, this medication will only be administered for 3 days per week.

Dose: Dasatinib in the form of Sprycel (Bristol Myers Squibb) 100 mg orally daily for 3 consecutive days. One (100 mg) tablet will be taken in the morning for each of the 3 days for three consecutive weeks followed by a four-week medication free period. This cycle will be repeated three times.

The placebo group will receive a matched number of placebo capsules according to this schedule.

#### **Quercetin**

Quercetin is a naturally occurring flavonoid known to inhibit PI3Kinase, other kinases, and mTOR pathways. Quercetin is present in many fruits, vegetables, and grains and is also used as an ingredient in supplements, beverages, or various types of foods.

Dose: Quercetin dehydrate capsules equating to 1000 mg total daily dosage will be administered orally. One (500 mg) capsule will be taken each morning and one (500 mg) capsule will be taken in the evening for each of the 3 days for three consecutive weeks followed by a four-week medication free period. This cycle will be repeated three times.

The placebo group will receive a matched number of placebo capsules according to this schedule.

This protocol is based on current preclinical studies and recent investigations published in *Aging Cell*<sup>25</sup>. In addition, we based our dosing on the recently published proof of principle studies in obesity/diabetic kidney disease and idiopathic pulmonary fibrosis - diseases characterized by accumulation of senescent cells<sup>34,36</sup>.

### 5.2 Use of co-intervention

Individuals with NAFLD and related liver fibrosis in the Netherlands are typically treated with lifestyle intervention. If an individual also has type 2 diabetes, then they are typically treated with metformin, and, depending on further glycaemic control (and treatment

adherence), with other oral glucose lowering drugs such as sulfonylureas, dipeptidylpeptidase-4 inhibitors, sodium glucose reuptake inhibitors, or subcutaneously injected insulin (either long- and/or short acting or a combination and /or mix of both). Sulfonylureas and insulin are associated with hypoglycemia and their dose will be carefully adjusted if needed. Especially because recently the suggestion that dasatinib is associated with improved glucose metabolism and decreased need for glucose lowering medication was further substantiated. Lifestyle programs are not paused.

Drugs listed as part of the exclusion criteria are not permitted during the course of treatment with dasatinib and quercetin. If individuals are required to initiate these medications, they will be removed from the study primarily due to risk of drug-drug interaction. Special attention is given to substances that either increase or decrease dasatinib plasma concentrations.

#### Active substances that may increase dasatinib plasma concentrations

In vitro studies indicate that dasatinib is a CYP3A4 substrate. Concomitant use of dasatinib and medicinal products or substances which potentially inhibit CYP3A4 (e.g., ketoconazole, itraconazole, erythromycin, clarithromycin, ritonavir, telithromycin, grapefruit juice) may increase exposure to dasatinib. Therefore, in patients receiving dasatinib, systemic administration of a potent CYP3A4 inhibitor is not recommended and is therefore an exclusion criterion.

#### Active substances that may decrease dasatinib plasma concentrations

Medicinal products that induce CYP3A4 activity such as rifampicin, dexamethasone, phenytoin, carbamazepine, phenobarbital or herbal preparations containing *Hypericum perforatum*, also known as St. John's Wort) may increase metabolism and decrease dasatinib plasma concentrations. Therefore, concomitant use of potent CYP3A4 inducers with dasatinib is not recommended.

#### Histamine-2 antagonists and proton pump inhibitors

Long-term suppression of gastric acid secretion by H2 antagonists or proton pump inhibitors (e.g., famotidine and omeprazole) is likely to reduce dasatinib exposure. The use of antacids should be considered in place of H2 antagonists or proton pump inhibitors in patients receiving dasatinib because antacids do not alter dasatinib concentrations.

## 6. INVESTIGATIONAL PRODUCT

### 6.1 Name and description of investigational product(s)

In this study, the combination of dasatinib a tyrosine kinase inhibitor and quercetin, a naturally-occurring flavonoid will be used.

### 6.2 Summary of findings from non-clinical studies

Since the discovery that the combination of dasatinib plus quercetin has the potential to selectively eliminate senescent cells, many non-clinical studies has been published. A selection of studies using dasatinib plus quercetin is shown in table 1.

**Table 1. selection of studies using dasatinib plus quercetin and with the established effects (D+Q)**

| Disorders and diseases                                                        | Pharmacologic agent                          | Phenotype of intervention                                                                                                                                                                                                                                        |
|-------------------------------------------------------------------------------|----------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Hepatic steatosis/cirrhosis/fibrosis <sup>37–40</sup>                         | D + Q <sup>37–40</sup>                       | Reduced hepatic steatosis and liver fibrosis                                                                                                                                                                                                                     |
| Diabetes/obesity/age-related lipodystrophy <sup>11,41–46</sup>                | D + Q <sup>41,44,46,47</sup>                 | Improved metabolic and adipose tissue function, reduced inflammation, improved adipogenesis, improved beta cell function                                                                                                                                         |
| Cardiac dysfunction <sup>25,48–50</sup>                                       | Q <sup>48</sup><br>D + Q <sup>25,49,50</sup> | Activation of resident cardiac progenitor cells and cardiomyocyte formation, alleviated myocardial hypertrophy and fibrosis, improved left ventricular ejection fraction, increase in myocardial vascularization, increased survival after myocardial infarction |
| Vascular hyporeactivity/calcification/arteriovenous fistulae <sup>49,51</sup> | D + Q <sup>49,51</sup>                       | Improved vasomotor function, reduced aortic calcification                                                                                                                                                                                                        |

| Disorders and diseases                                                                                         | Pharmacologic agent                       | Phenotype of intervention                                                                                                                                                                                                  |
|----------------------------------------------------------------------------------------------------------------|-------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Frailty/sarcopenia/muscular dystrophy/fibrodysplasia <sup>25,35,52</sup>                                       | D + Q <sup>25,35,52</sup>                 | Improved and delayed age-associated physical dysfunction, extended median and maximum lifespan                                                                                                                             |
| Age-related impairment of muscle hypertrophy after resistance exercise <sup>53</sup>                           | D + Q <sup>53</sup>                       | Improved muscle growth                                                                                                                                                                                                     |
| Renal dysfunction <sup>54–56</sup>                                                                             | D + Q <sup>54,55</sup><br>Q <sup>55</sup> | Improved renal function, reduced damage and fibrosis                                                                                                                                                                       |
| Osteoporosis/osteoarthritis/rheumatoid arthritis/intervertebral disc disease/fracture healing <sup>57,58</sup> | D + Q <sup>57,58</sup>                    | Attenuated the development of osteoarthritis, reduced pain and increased cartilage development, higher bone mass and strength and better bone microarchitecture, attenuated age-dependent intervertebral disc degeneration |
| Idiopathic lung fibrosis <sup>35</sup>                                                                         | D + Q                                     | Improved pulmonary function and reduced fibrosis                                                                                                                                                                           |
| Intestinal inflammation/microbiome <sup>59</sup>                                                               | D + Q                                     | Reduced inflammation and microbial dysbiosis                                                                                                                                                                               |
| Lifespan/healthspan <sup>52,60</sup>                                                                           | D + Q                                     | Delayed onset and progression of age-related pathologies, reduced frailty increased maximum lifespan                                                                                                                       |
| COVID-19 (refs. <sup>61,62</sup> )                                                                             | D + Q                                     | Reduced mortality, reduced inflammation, increased antiviral antibodies                                                                                                                                                    |

All in all, these studies show that the senolytic drug combination dasatinib plus quercetin has strong potential to restore progenitor dysfunction, attenuate tissue inflammation and alleviate age- and disease-related metabolic dysfunction across cell types and tissues.

In the summary of product characteristics for dasatinib an overview of the pre-clinical safety data can be found in section 5.3 at page 71-72.

[https://www.ema.europa.eu/en/documents/product-information/sprycel-epar-product-information\\_en.pdf](https://www.ema.europa.eu/en/documents/product-information/sprycel-epar-product-information_en.pdf)

### 6.3 Summary of findings from clinical studies

Dasatinib has been used for a few decades and has been approved as a prescription drug in the EU for various cancers and for fibrotic diseases (scleroderma, progressive systemic sclerosis), usually at doses of 100 mg/day for life. The intermittent treatment strategy in combination with quercetin to target cellular senescence for various diseases was considered a completely new therapeutic paradigm.

The first-in-human trial of senolytics was of dasatinib plus quercetin for patients with idiopathic pulmonary fibrosis ( $N = 14$  patients) in an open-label study of intermittent dasatinib plus quercetin (3 days/week over 3 weeks)<sup>34</sup>. Physical function was evaluated by testing 6-minute walk distance, 4-metre gait speed and chair-stands time. These assessments were significantly and clinically meaningfully improved by dasatinib plus quercetin ( $P < 0.05$ ). Furthermore, correlations were found between change in function and change in SASP-related pro-inflammatory cytokines, matrix-remodeling proteases and micro-RNAs markers;  $r \geq 0.50$ ). This pilot study supports the feasibility of further clinical trials using senolytics. It provided first evidence that senolytics can alleviate physical dysfunction.

In a preliminary report from an early, open-label clinical trial of 3 days, oral dasatinib plus quercetin administration in individuals with diabetes complicated by renal dysfunction, evidence for target engagement was found<sup>36</sup>. Compared to baseline, p16<sup>INK4a+</sup> and SA- $\beta$ gal<sup>+</sup> senescent cells had decreased in adipose tissue of individuals 11 days after the last dose of dasatinib plus quercetin. Furthermore, dasatinib plus quercetin decreased adipose tissue inflammation as evident by reduced macrophage number and reduced formation of crown-like structures. A composite score of blood SASP factors was also decreased by dasatinib plus quercetin in these individuals. Thus, as in mice, dasatinib plus quercetin can successfully decrease senescent cell burden in humans.

Several clinical trials of senolytics are currently underway or planned (Table 2, page 25-2). These include Phase I or II trials of dasatinib plus quercetin for idiopathic pulmonary fibrosis, age-related osteoporosis, age-related frailty, frailty in bone marrow transplant survivors with an accelerated aging-like syndrome, frailty in childhood cancer survivors in

their late 30's and 40's also with an accelerated aging-like syndrome, the ongoing trial in diabetic chronic kidney disease, Alzheimer's disease (Tau<sup>+</sup>, symptomatic [clinical dementia rating 1]) and trials for improving outcomes after transplanting organs from old donors.

Findings from clinical studies using only dasatinib can be found in the Summary of Product Characteristics (SPC) on page 20-32. It is important to note that unlike standard treatment regimens also noted in the SPC, in this trial, this medication will only be administered for 3 days per week.

[https://www.ema.europa.eu/en/documents/product-information/sprycel-epar-product-information\\_en.pdf](https://www.ema.europa.eu/en/documents/product-information/sprycel-epar-product-information_en.pdf)

**Table 2. Overview of ongoing studies using dasatinib plus quercetin (D+Q).**

| Study title                                                                           | Senolytic | Study design                                                                                | Identifier                                                  | Status                                |
|---------------------------------------------------------------------------------------|-----------|---------------------------------------------------------------------------------------------|-------------------------------------------------------------|---------------------------------------|
| Targeting pro-inflammatory cells in idiopathic pulmonary fibrosis: a human trial      | D + Q     | Phase 1, randomized, open-label                                                             | <a href="#">NCT02874989</a>                                 | Completed                             |
| Senescence in chronic kidney disease                                                  | D + Q     | Phase 2, randomized, open-label                                                             | <a href="#">NCT02848131</a>                                 | Current, preliminary report published |
| Hematopoietic stem cell transplant survivors study (HTSS)                             | D + Q     | Randomized, open-label                                                                      | <a href="#">NCT02652052</a>                                 | Current                               |
| ALSENLITE: senolytics for Alzheimer's disease                                         | D + Q     | Phase 1/2, open-label                                                                       | <a href="#">NCT04785300</a>                                 | Current                               |
| Senolytic therapy to modulate the progression of Alzheimer's disease (SToMP-AD) study | D + Q     | Phase 1/2, open-label, pilot study<br>Phase 2, randomized, double-blind, placebo-controlled | <a href="#">NCT04063124</a> and <a href="#">NCT04685590</a> | Current                               |
| Senolytics to improve cognition and mobility in older                                 | D + Q     | Single-arm, open-label, pre-post pilot study                                                | Pending                                                     | Pending                               |

| Study title                                                                                                      | Senolytic | Study design                                         | Identifier                         | Status  |
|------------------------------------------------------------------------------------------------------------------|-----------|------------------------------------------------------|------------------------------------|---------|
| adults at risk of Alzheimer's disease                                                                            |           |                                                      |                                    |         |
| An open-label intervention trial to reduce senescence and improve frailty in adult survivors of childhood cancer | D + Q;    | Phase 2, randomized, open-label                      | <a href="#"><u>NCT04733534</u></a> | Current |
| Targeting cellular senescence with senolytics to improve skeletal health in older humans                         | D + Q;    | Phase 2, randomized, open-label                      | <a href="#"><u>NCT04313634</u></a> | Current |
| Quercetin in coronary artery bypass surgery (Q-CABG)                                                             | Q         | Phase 2, randomized double-blind, placebo-controlled | <a href="#"><u>NCT04907253</u></a> | Current |

## 6.4 Summary of known and potential risks and benefits

Worldwide, hundreds of individuals have been enrolled in senolytic clinical trials using dasatinib plus quercetin for a variety of conditions. Importantly, severe or serious side effects have so far not been reported. Of interest, in contrast to other tyrosine kinase inhibitors, dasatinib usage is not associated with an increased risk of hepatotoxicity<sup>16</sup>. Recently the suggestion that dasatinib is associated with improved glucose metabolism and decreased need for glucose lowering medication was further substantiated<sup>17</sup>. Side-effects of dasatinib include abdominal pain, vomiting, diarrhea, musculoskeletal pain, tiredness, and myelosuppression. Another side effect, which may occur after months of continuous use, include peripheral edema and pleural effusions. Importantly, it has been described that a twice daily dasatinib regimen, as well as a history of cardiac or autoimmune disease, are suspected as predisposing factors<sup>18,19</sup>. To minimize such risks, known predisposing factors were included in the exclusion criteria for the present RCT. In addition, pleural effusion is driven by a reactive oxygen species (ROS) dependent mechanism. Of interest, not only are these side effects reversible, but a recent study showed that the increased endothelial permeability, which drives the side-effects can be counteracted, using antioxidants such as quercetin<sup>20</sup>.

According to the previous studies using the intermittent dosing strategy with the combination of dasatinib plus quercetin we divided the side-effects bases on whether they occur frequent or not.

Frequent side effects:

Gastro-intestinal and appetite: Nausea, vomiting, constipation, diarrhea, abdominal pain, heartburn, change in appetite, weight change.

General health & well-being: Headache, skin rash and dry eyes, sleeplessness, fatigue and general unwellness.

Less frequent side effects with the intermittent dosing strategy: Fever, infections (viral, bacterial, fungal), Lowering of white and/or red blood cells, or platelets, allergy to either drug, fluid retention. arrhythmias, QT time extension, electrolyte alterations, fluid retention, pleural effusions.

## **6.5 Description and justification of route of administration and dosage**

Both dasatinib as well as quercetin and the matched placebo tablets are taken orally.

## **6.6 Dosages, dosage modifications and method of administration**

Intervention arm: Dasatinib 100 mg, once daily + quercetin 1000 mg.

Placebo arm: placebo tablet of dasatinib + placebo quercetin.

## **6.7 Preparation and labelling of Investigational Medicinal Product**

To meet the quality requirements for research with trial medication according to Good Clinical Practice (GCP) and Good Distribution Practice (GDP), this trial has been registered at the “Knowledge Centre Drug Research” of the AMC. The import, packaging, labelling, storage and issuing of study medication will be guaranteed by the Clinical trial Pharmacy of the AMC.

## **6.8 Drug accountability**

To meet the quality requirements for research with trial medication according to Good Clinical Practice (GCP) and Good Distribution Practice (GDP), this trial has been registered at the “Knowledge Centre Drug Research” of the AMC. The import, packaging, labelling, storage and issuing of study medication will be guaranteed by the Clinical trial Pharmacy of the AMC for the dasatinib and matched placebo tablets.

Dasatinib will be delivered by Bristol Myers Squibb to the trial pharmacy of the

Amsterdam UMC, location AMC. The Amsterdam UMC, location AMC will recapsulate and repack the dasatinib into the correct dose per patient and label the packages with the randomization numbers and other information required by GMP. The capsules are dispersed by the pharmacy of The Amsterdam UMC, location AMC in accordance with GMP guidelines, packed in the correct dosage and labelled with the randomization numbers.

For the quercetin and matched placebo tablets will be obtained from Solipharma bv who are collaborating with Frutarom, JRS Pharma, Capsugel. As shown in attachment D, Solipharma and collaborators has all necessary qualities to produce and distribute quercetin and has an official certificate of GMP compliance of the manufactures. Quercetin and placebo matched tablets will be stored at the clinical trial pharmacy of the AMC.

Once all medication is obtained, the clinical trial pharmacy will store all the medication, repack into the correct dose per participant, label the packages with the randomization numbers thereby ensuring the correct distribution. In case the medication is not used by the participants, the investigator will return the medication to the clinical trial pharmacy where it will be noted and destroyed once the study is finished.

## **7. NON-INVESTIGATIONAL PRODUCT**

n/a

## **8. METHODS**

### **8.1 Study parameters/endpoints**

#### **8.1.1 Main study parameter/endpoint**

The primary endpoint is the binary outcome improvement of fibrosis with at least 1-point without worsening of fibrosis and NAFLD score based on histology after 21 weeks (yes/no). Individuals will be labeled as responder or non-responder.

#### **8.1.2 Secondary study parameters/endpoints**

##### Liver-related histological parameters and high dimensional data

- Mean change in number of senescent cells at baseline and end of treatment (week 21)
- Percent of patients with reversal of NAFLD (Steatosis without ballooning and with or without mild inflammation) and no worsening of fibrosis) from baseline to end of treatment (week 21).
- Global hepatic mRNA expression baseline to end of treatment (week 21).

Change from baseline to week 21 in:

- NAFLD activity score (NAS)

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- Activity component of steatosis-activity-fibrosis (SAF) score: steatosis -1 point, lobular inflammation -1 point, ballooning -1 point  
EPOS score a 7 item scoring system (0-6) which represent the seven stages of fibrosis.

#### Biomarkers of NAFLD disease

Change from baseline to week 21 in:

- Fibrosis-4 score (Fib-4 score) and NAFLD Fibrosis Score (NFS)
- Liver enzymes and synthesis function: ALT, AST and yGT, albumin, INR
- Liver stiffness and liver steatosis (with controlled attenuation parameter) measurement by Fibroscan

#### Glucose metabolism related parameters

Change from baseline to 21 weeks in:

- Glycosylated haemoglobin type A1c (HbA<sub>1c</sub>), Fasting plasma glucose (FPG), Fasting insulin and glucagon
- Homeostatic model assessment of insulin resistance (HOMA-IR)
- Glucose day curve (Freestyle libre)

#### Patient reported outcomes

Change from baseline to 21 weeks in:

- RAND-36 and EQ-5D-5L: physical and mental component summary scores and scores on the individual sub-domains: Physical functioning, role functioning, bodily pain, general health, vitality, social functioning, role emotional and mental health.

#### Safety endpoints

- Number of treatment-emergent adverse events during the trial
- Number of treatment-emergent myelosuppression
- Number of treatment emergent infections
- Number of subjects discontinuing treatment due to gastrointestinal adverse events

#### Change from baseline to 21 weeks in:

- Pulse and ECG
- Physical examination
- Haematology (haemoglobin, thrombocytes, erythrocytes, leucocytes, differential count)
- Biochemistry (creatinine, creatinine phosphokinase, urea, bilirubin (total), alkaline phosphatase, ferritin, sodium, potassium, calcium.
- Fecal microbiota composition (morning stool and bile acids)

## **8.2 Randomisation, blinding and treatment allocation**

Randomization will take place after all the inclusion criteria are met and after the participant has granted written informed consent to participate in the study. The participant will be randomized into one of the two treatment arms. The randomization will be performed in blocks of 4 and is not stratified; participant will be assigned randomly to one of the two groups via CASTOR EDC. CASTOR EDC is used as electronic case record file and is compliant with the 21 CFR part 11, ICH e6 GCP, GDPR and HIPAA and is ISO27001 and ISO9001 certified.

In CASTOR, double randomization is performed using two different randomization rights that can be assigned individually to every user. The randomization right allows investigators to distribute participants into different intervention groups. Users with the randomization right will be able to see the date of randomization and who performed it. The actual randomization number and allocation will remain hidden for the investigators until after the completion of the trial.

Only users with the 'view randomization' right can see to which group a participant (record) has been allocated. In this case, the head of the clinical Pharmacy of the AMC and the DSMB. Therefore, only the pharmacist has access to the allocation data. In medical charts the randomization number is recorded upon providing written informed consent. This ensures that all study procedures and samples obtained (i.e., biopsy scores, liver elastography results, anthropometric data, etc.) are blinded for all investigators. In case of a suspected unexpected serious adverse reaction (SUSAR) treatment with the investigational product is discontinued and the debinding procedure is initiated.

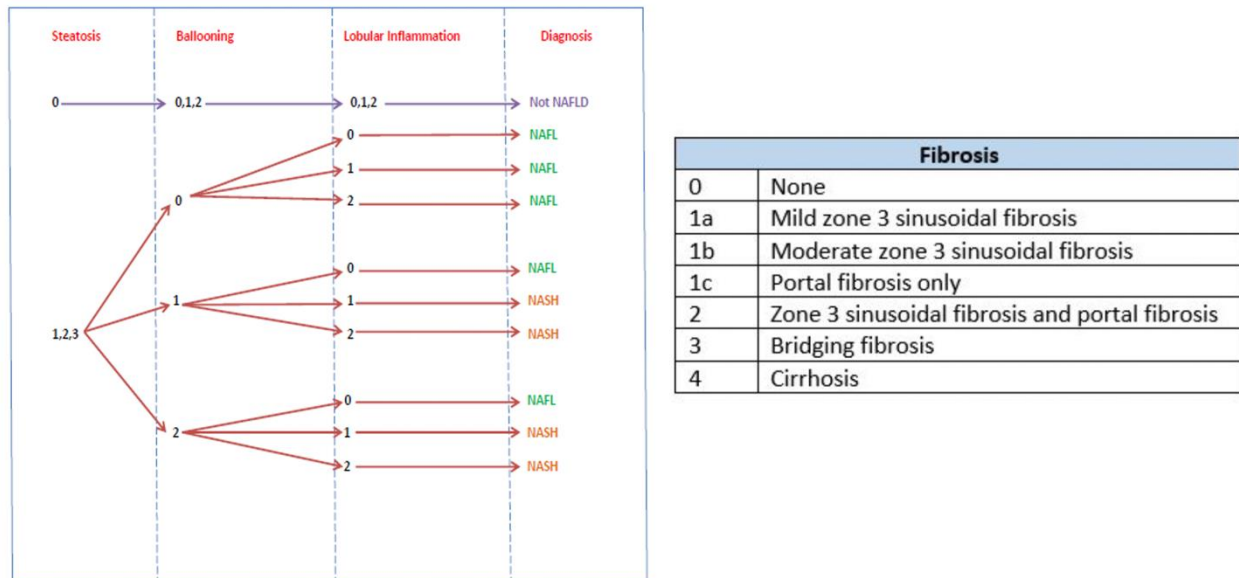
When debinding is considered necessary by the clinicians involved, the study coordinator is notified. The pharmacist and the coordinating investigator will decide whether it is necessary to break the randomization code. If so, the independent physician can break the code and discuss the results with the attending clinician of the participant. This way, blinding of the investigators is not disrupted.

## **8.3 Study procedures**

### **8.3.1 The ultrasound-guided liver biopsy**

The ultrasound-guided liver biopsy will be performed by either an experienced intervention radiologist from the AMC Department of Radiology or hepatologist from the AMC department of Gastroenterology (see METC 2017\_311, 2019\_61 and 2022\_236). After an overnight fast, the patient will be in supine position and using ultrasound a non-vascularized segment of the right liver lobe will be identified. A needle (14 to 19 gauge) will be used for the biopsy, and during the biopsy the patient is asked to hold their breath. A bandage will be put on the puncture site. The test generally takes 15 to 30 minutes. The patient will be instructed to lie on his/her right side for 1 to 4 hours after the test. Pulse, blood pressure, and temperature will be checked after the biopsy. All biopsy samples will be performed by an experienced radiologist or hepatologist and directly frozen in liquid nitrogen and stored at -80C until analysis. When performed ultrasound-guided, percutaneous liver biopsy is a safe method with a low complication rate (usually self-

limiting bleeding) of less than 0.1% which in our study with a maximum of 2 biopsies per person would in theory implicate that none of the individuals will have a complication. The histopathological diagnosis of NAFLD as well as fibrosis will be assessed using the SAF (Steatosis, Activity, Fibrosis) scoring system according to the following flowchart.



### Flowchart of Steatosis Activity and Fibrosis score.

From the obtained liver biopsies, one piece will be paraffin embedded for histology and one will be put on liquid nitrogen for RNA sequencing as previously described by our group<sup>14</sup>.

### 8.3.2 Liver elastography (Fibroscan)

The Fibroscan device works by measuring shear wave velocity. In this technique, a 50-MHz wave is passed into the liver from a small transducer on the end of an ultrasound probe. The probe also has a transducer on the end that can measure the velocity of the shear wave (in meters per second) as this wave passes through the liver. The shear wave velocity can then be converted into liver stiffness, which is expressed in kilopascals (table 3). The Cutoffs in Table correspond with the fibrosis score based on histology according to the SAF scores.

**Table 3: Recommended values for fibrosis staging in NAFLD**

|            | F0-F1 | F2   | F3  | F4    |
|------------|-------|------|-----|-------|
| kilopascal | ≤7.5  | ≥7.5 | ≥10 | ≥14.0 |

### **8.3.3 Blood samples**

During every visit and after an overnight fast, in all subjects 30ml of venous blood samples will be taken to look at inflammatory markers (CRP, ferritin) liver function tests (alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), albumin, and bilirubin tests. Moreover, blood will be taken to measure glucose, insulin, glucagon and hemoglobin A1c. To be aware of myelosuppression due to administration of dasatinib, during screening and after the first treatment cycle, complete blood count will be examined. In addition, HCG will be measured to exclude pregnancy.

### **8.3.4 Electrocardiography**

An electrocardiogram (ECG) will be performed to detect any arrhythmias at screening and prior to each treatment cycle. In total 4 ECG's will be made and interpreted by an experienced clinical trained for reading ECG's.

### **8.3.5 Measurement of blood pressure**

Brachial blood pressure is measured using a validated semi-automated oscillometric device (Nexfin Omron 705it), while seated and after 5 minutes rest in a quiet room, 3 times at 1-minute intervals (the average of the latter two measurements will be used). The first visit blood pressure is recorded at both arms. If the difference in systolic blood pressure >10mmHg, the arm with the highest recording will be taken for future readings. In other cases, the non-dominant arm will be used for future readings. The lowest of the three measurements is recorded as representative of brachial blood pressure.

### **8.3.6 Questionnaires**

Prior to the start and after the treatment, participants will be asked to fill in two questionnaires: RAND-36 and EQ-5D-5L. With these questionnaires physical and mental component summary scores and scores on the individual sub-domains: Physical functioning, role functioning, bodily pain, general health, vitality, social functioning, role emotional and mental health are collected.

### **8.3.7 Morning stool**

Morning stool samples will be collected to determine gut microbiota composition by whole genome sequencing. Samples will be taken by collection on toilet paper (by patient him/herself wearing gloves), divided over 3 eppendorfs and directly frozen in fridge at home (-20°C). Samples will be transported to the hospital on icepacks. At the hospital, all samples will be stored at -80°C. The samples will be analyzed at EVG (laboratory of prof. Nieuwdorp at AMC) by 16s sequencing (Illumina MiSeq) which is developed for exact and sensitive enumeration of bacterial population.

### **8.3.8 Freestyle libre**

The Freestyle libre is a wireless system using a sensor to measure glucose levels during the day and night. The sensor is attached to the skin on the backside of the arm and measures glucose in the subcutaneous tissue fluid. Unlike other glucose measurements,

the Freestyle libre does not require a finger prick. Patients will receive a Freestyle libre during visit 2 and the device can be read during every visit.

### 8.3.9 Dietary intake (3 days on paper)

Every participant is asked to keep track of their dietary intake during 3 days online at [mijn.voedingscentrum.nl/nl/eetmeter](http://mijn.voedingscentrum.nl/nl/eetmeter). A researcher will impute this in the electronic case record file database (Castor)

**Table 4. Content and duration of the study visits**

|                  | Visit                                                                            | Content of visit                                                                                                                                                                                                                                                                                           |
|------------------|----------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Before treatment | <b><u>First interview</u></b><br>(by phone)                                      | First explanation of the study<br>Patients can express their interest                                                                                                                                                                                                                                      |
|                  | <b><u>Visit 1</u></b><br>Screening<br>Week -3<br>Duration: approximately 4 hours | Explanation<br>study Signing<br>ICF Screening: <ul style="list-style-type: none"> <li>▪ Fasting blood sampling + HCG</li> <li>▪ ECG</li> <li>▪ Physical examination</li> <li>▪ Blood pressure</li> <li>▪ Ultrasound-guided liver biopsy (if needed)</li> </ul>                                             |
|                  | <b><u>Visit 2</u></b><br>Week -2<br>Duration: approximately 1 hour               | <ul style="list-style-type: none"> <li>• Fasting blood sampling + HCG</li> <li>• Physical examination</li> <li>• Fibroscan</li> <li>• Reach out the first batch of medication</li> <li>• Feces collection</li> <li>• Dietary intake list</li> <li>• Handing out the Freestyle libre</li> </ul>             |
| During treatment | <b><u>Visit 3</u></b><br>Week + 7<br>Duration: approximately 1 hour              | <ul style="list-style-type: none"> <li>▪ Fasting blood sampling + HCG</li> <li>▪ Physical examination</li> <li>▪ ECG</li> <li>▪ Fibroscan</li> <li>▪ Feces collection</li> <li>▪ Dietary intake list</li> <li>▪ Reading out Freestyle libre</li> <li>▪ Reach out the second batch of medication</li> </ul> |

|                 |                                                                      |                                                                                                                                                                                                                                                                                                                            |
|-----------------|----------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                 | <b><u>Visit 4</u></b><br>Week + 14<br>Duration: approximately 1 hour | <ul style="list-style-type: none"> <li>▪ Fasting blood sampling + HCG</li> <li>▪ Physical examination</li> <li>▪ ECG</li> <li>▪ Fibroscan</li> <li>▪ Fecec collecion</li> <li>▪ Dietary intake list</li> <li>▪ Handing out Freestyle libre Reach out the third batch of medication</li> </ul>                              |
| After treatment | <b><u>Visit 5</u></b><br>Week +21<br>Duration: approximately 4 hours | <ul style="list-style-type: none"> <li>▪ Fasting blood sampling + HCG</li> <li>▪ Physical examination</li> <li>▪ Fibroscan</li> <li>▪ Blood pressure</li> <li>▪ Ultrasound-guided liver biopsy</li> <li>▪ Questionnaires Feces collection</li> <li>▪ Dietary intake list</li> <li>▪ Reading out Freestyle libre</li> </ul> |

#### 8.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

In the case of withdrawal due to pregnancy, the patient will be followed until outcome, i.e. at least on a quarterly basis for follow-up evaluation of the pregnancy, foetus, delivery and new born. In the case of withdrawal due to an AE the patient will be followed until resolution of the AE, or until in the opinion of the Investigator the event has stabilized, and the patient is referred to their primary physician for appropriate management of the ongoing event. Reasonable efforts will be made to contact a patient who fails to attend any follow-up appointments, in order to ensure that he/she is in satisfactory health.

#### 8.5 Replacement of individual subjects after withdrawal

An individual subject who withdraws from the study will be replaced by a new subject to meet the sample size. If a subject discontinues from the study prematurely the reason, if given, must be fully evaluated and recorded appropriately in source documentation and the CRF. We expect a drop-out of 10%, hence 33 individuals need to be recruited.

#### 8.6 Follow-up of subjects withdrawn from treatment

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Should a subject request or decide to withdraw from the study, all efforts will be made to complete and report the observations as thoroughly as possible up to the date of withdrawal, and for subjects who are withdrawn.

#### **8.7 Premature termination of the study**

If the trial is prematurely terminated or suspended for any reason, the investigator should promptly inform the trial subjects and should assure appropriate therapy and follow-up. The sponsor, IRB/IEC and regulatory authority (ies) should be notified

## 9. SAFETY REPORTING

### 9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardies subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

### 9.2 AEs, SAEs and SUSARs

#### 9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to [the investigational product / trial procedure/ the experimental intervention]. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

#### 9.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

**Pregnancy:** The occurrence of a pregnancy of a study patient or a partner of a study patient discovered during IMP administration or within 1 week after last administration of the IMP, is to be communicated to the Sponsor in an expedited manner with the same procedure and timelines as for SAEs independently from the occurrence of an AE.

Specific follow-up of the event will be requested by the Sponsor until pregnancy outcome, at least on a quarterly basis as described in paragraph 8.4.

The investigator will report all SAEs to the sponsor without undue delay after obtaining knowledge of the events, except for the following SAEs:

The sponsor will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 7 days of first knowledge for SAEs that result in Protocol TRUTH versie 3.3 d.d. 10-6-2024 M. Koning, afd. vasculaire geneeskunde Amsterdam UMC, locatie AMC

death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

### **9.2.3 Suspected unexpected serious adverse reactions (SUSARs)**

Adverse reactions are all untoward and unintended responses to an investigational product related to any dose administered.

Unexpected adverse reactions are SUSARs if the following three conditions are met:

1. the event must be serious (see chapter 9.2.2);
2. there must be a certain degree of probability that the event is a harmful and an undesirable reaction to the medicinal product under investigation, regardless of the administered dose;
3. the adverse reaction must be unexpected, that is to say, the nature and severity of the adverse reaction are not in agreement with the product information as recorded in:

- Summary of Product Characteristics (SPC) for an authorised medicinal product;
- Investigator's Brochure for an unauthorised medicinal product.

The sponsor will report expedited the following SUSARs through the web portal *ToetsingOnline* to the METC:

- SUSARs that have arisen in the clinical trial that was assessed by the METC;
- SUSARs that have arisen in other clinical trials of the same sponsor and with the same medicinal product, and that could have consequences for the safety of the subjects involved in the clinical trial that was assessed by the METC.

The remaining SUSARs are recorded in an overview list (line-listing) that will be submitted once every half year to the METC. This line-listing provides an overview of all SUSARs from the study medicine, accompanied by a brief report highlighting the main points of concern.

The expedited reporting of SUSARs through the web portal Eudravigilance or *ToetsingOnline* is sufficient as notification to the competent authority.

The sponsor will report expedited all SUSARs to the competent authorities in other Member States, according to the requirements of the Member States.

The expedited reporting will occur not later than 15 days after the sponsor has first knowledge of the adverse reactions. For fatal or life threatening cases the term will be maximal 7 days for a preliminary report with another 8 days for completion of the report. When blinding is considered necessary by the clinicians involved in the treatment of the patient, the study coordinator is notified. The pharmacist and the coordinating investigator will decide whether it is necessary to break the randomization code. If so, the independent physician can break the code and discuss the results with the attending clinician of the patient. This way, blinding of the investigators is not disrupted.

### **9.3 Annual safety report**

In addition to the expedited reporting of SUSARs, the sponsor will submit, once a year throughout the clinical trial, a safety report to the accredited METC, competent authority, and competent authorities of the concerned Member States.

This safety report consists of:

- a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study;
- a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the medicine under investigation.

### **9.4 Follow-up of adverse events**

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported till end of study within the Netherlands, as defined in the protocol

### **9.5 [Data Safety Monitoring Board (DSMB) / Safety Committee]**

An independent DSMB is installed (comprising chair: prof dr J.B.L. Hoekstra internist, prof dr HR Buller, internist at AMC and dr B.A.Hutten, statistician Amsterdam UMC) before the trial will be initiated.

### **9.6 COVID regulations**

This study will adhere to the general guidelines regarding the safe practice of clinical studies during the COVID19 pandemic. Participants will be asked whether they have COVID19 related complaints prior to all study visits and adequate social distancing and protective wear will be maintained throughout all study visits whenever possible.

## 10. STATISTICAL ANALYSIS

If necessary, a statistical analysis plan (SAP) may be written in addition to the protocol, including a more technical and detailed elaboration of the statistical analyses. The SAP will be finalized before database lock.

The estimand addressing the primary objective is defined as the de-facto (effectiveness) treatment effect on a 1-point improvement in NAFLD-related fibrosis without worsening in fibrosis and NAFLD for all randomized subjects after 21 weeks. All post-baseline scheduled visit data will be included in the statistical analysis, including data collected after discontinuation of trial product. The chosen estimand assesses the difference in a 1-point fibrosis score in a future population that results from initiating treatment with dasatinib plus quercetin as compared to placebo. Generalization of this estimand depends among other things on the extent to which treatment adherence in this trial reflects clinical practice.

The statistical analyses will in general consist of the following pairwise treatment comparison:

Dasatinib 100 mg + quercetin 1000 mg versus placebo

The results of the comparison will be presented as estimated treatment contrasts together with two-sided 95% confidence intervals and p-values corresponding to two-sided tests of no difference.

The full analysis set (FAS) will be used in the analysis of the efficacy endpoints whereas the safety analysis set (SAS) will be used for the safety endpoints.

The FAS includes all randomized subjects. Subjects in the FAS will contribute to the evaluation “as randomized”.

The SAS includes all subjects receiving at least one dose of randomized treatment. Subjects in the SAS will contribute to the evaluation “as treated”.

The baseline measurement is defined as the latest available measurement at or prior to the randomization visit. An exception is made for identifying abnormal laboratory findings (including ALT, AST, GGT, bilirubin and INR) in which case the baseline value is defined as the mean of the available measurements at the screening and randomization visits. Before data are locked for statistical analysis, a review of all data will take place. Any decision to exclude a subject or single observations from the statistical analysis is the joint responsibility of the trial statistician, the principal investigator and investigators. Exclusion of data from analyses will be used restrictively and normally no data should be excluded from the FAS. If any data is excluded, the decision will be justified and documented. The subjects and observations excluded from analysis sets, and the reason for their exclusion, will be described in the clinical trial report.

### 10.1 Primary study parameter(s)

The primary endpoint is the binary outcome improvement of fibrosis with at least 1-point without worsening of fibrosis and NAFLD score based on histology after 21 weeks (yes/no). Individuals will be labeled as responder or non-responder. The difference between responders and non-responders will be compared using the chi-square test.

### 10.2 Secondary study parameter(s)

The following secondary histological endpoint will be analyzed:

- Percent of patients with reversal of NAFLD (Steatosis without ballooning and with or without mild inflammation) and no worsening of fibrosis) from baseline to end of treatment (week 21) noted as responders or non-responders. This analysis will be performed using the chi-square test.
- NAFLD activity score (NAS)
  - Steatosis (0-3)
  - Lobular inflammation (0-3)
  - Hepatocyte ballooning (0-2)
- EPOS 7 system score system (0-6)

From baseline to end of treatment (week 21) Individuals will be labeled as responder or non-responder. The difference between responders and non-responders will be compared using the chi-square test

- Activity component of steatosis-activity-fibrosis (SAF) score:
  - lobular inflammation 2 point,
  - hepatocyte ballooning 2 point

The activity component of the SAF score is defined as the unweighted sum of hepatocyte ballooning and lobular inflammation. The definition of the lobular inflammation score is modified in this calculation so that the scores 2 and 3 on the original scale are merged to a score 2. The possible range of the sum is thus 0 to 4. For all scores, a higher value indicates a more severe state of disease.

The histological feature scores will be analyzed by an ordered logistic regression model (also known as a proportional odds model) with the score at week 21 as response; treatment, baseline diabetes status, baseline fibrosis stage, diabetes-by-fibrosis interaction and corresponding baseline score as factors; and baseline body weight as a covariate. The results will be presented as an estimate of the cumulative odds ratio for each treatment comparison together with the associated 95% confidence intervals and p-values.

The mean change within the treatment group in number of senescent cells and end of treatment (week 21) will be assessed using a paired t-test .

### Biomarkers of NAFLD disease

Analyses will be performed for the change from baseline to week 21 in the following biomarkers for NAFLD disease:

- Fibrosis-4 score (Fib-4 score) and NAFLD Fibrosis Score (NFS)
- Liver enzymes and synthesis function: ALT, AST and  $\gamma$ GT, albumin, INR
- Liver stiffness and liver steatosis (with controlled attenuation parameter) measurement by Fibroscan

The main analysis of each of the biomarkers will be analysis of covariance (ANCOVA). An ANCOVA model with baseline diabetes status, baseline fibrosis stage and diabetes-by-fibrosis interaction as factors and baseline body weight and baseline value of the corresponding biomarker as covariates is fitted to the change from baseline to 21 weeks.

A supportive on-treatment analysis using a mixed model for repeated measurements (MMRM) will be performed for each of the biomarkers. In this model, all scheduled post-baseline measurements taken during the individual subject's on-treatment period will enter as response; treatment, baseline diabetes status, baseline fibrosis stage and diabetes-by-fibrosis interaction will be included as factors; and baseline body weight and baseline value of the corresponding biomarker will be included as covariates. All factors and covariates will be nested within visit and an unstructured covariance matrix for measurements within subject will be employed. There will be no explicit imputation of missing values. As for the main analysis, the estimated treatment differences at week 21 with associated 95% confidence intervals and p-values will be presented.

Additional exploratory analyses will be conducted to evaluate the performance of the biomarkers as predictors of NAFLD (yes/no), fibrosis (stage  $\geq 2$ ,  $\geq 3$  and 4, respectively) and/or steatosis (score  $\geq 1$ ,  $\geq 2$  and 3, respectively) as applicable using the liver biopsy as the gold-standard reference. Separate analyses will be performed at baseline and week 21 for each predictor. A measurement will be classified as "positive" if the value of the predictor exceeds a chosen cut-point. The performance will be illustrated by the receiver operating characteristic (ROC) curve which describes the relationship of the true positive rate (sensitivity) versus the false positive rate ( $1 - \text{specificity}$ ) as one moves the cut-point. The area under the ROC curve will be used to summarize the overall performance of a predictor and the optimal cut-point will be determined by finding the maximum of Youden's index ( $\text{sensitivity} + \text{specificity} - 1$ ). In addition, new algorithms may be explored by constructing a logistic regression model which includes a large set of potential predictors and then selecting the most useful ones using stepwise backward elimination.

#### Glucose metabolism related parameters

The secondary endpoints related to glucose metabolism are defined as change from baseline to 21 weeks in:

- Glycosylated haemoglobin type A1c (HbA<sub>1c</sub>), Fasting plasma glucose (FPG), Fasting insulin and glucagon
- Homeostatic model assessment of insulin resistance (HOMA-IR)
- Glucose variability (Freestyle libre)
- Blood pressure

These endpoints will be analyzed using the same type of ANCOVA with multiple imputation (MI) as for the biomarkers. Supportive on-treatment analyses will be performed based on MMRM in the same way as for the biomarkers with factors and covariates specified as in the ANCOVA model.

#### Patient reported outcomes

The results from RAND-36 and EQ-5D-5 questionnaire will be analyzed as the change from baseline to 21 weeks in overall mental and physical scores, respectively, as well as the change in each of the 8 domains:

- Physical functioning
- Role functioning
- Bodily pain
- General health
- Vitality
- Social functioning
- Role emotional
- Mental health

The change in score will be analyzed as a continuous endpoint using the same type of ANCOVA with MI as for the biomarkers with treatment, baseline diabetes status, baseline fibrosis stage and diabetes-by-fibrosis interaction as factors and corresponding baseline score as a single covariate. Supportive on-treatment analyses will be performed based on MMRM in the same way as for the biomarkers with factors and covariates specified as in the ANCOVA model.

#### Pulse

Will be summarized by descriptive statistics and analyzed using the same type of MMRM as for the on-treatment analyses of the biomarkers with treatment, baseline diabetes status, baseline fibrosis stage and diabetes-by-fibrosis interaction as factors and baseline pulse as a covariate.

#### ECG

ECG will be described by summarizing the number and percentage of subjects with normal and abnormal readings (separated into clinically significant and not clinically significant). The summaries will be presented by visit and as shift tables from baseline to week 21.

#### Physical examination

The results of the physical examination will be summarized descriptively in the same way as ECG.

#### Laboratory assessments

The haematology and biochemistry parameters will be summarized and evaluated by descriptive statistics.

#### Fecal microbiota composition

Statistical analysis for the microbiome will be performed on rarefied count. Beta diversity associations will be tested using PERMANOVA analysis. Since we collect feces on multiple moments we will also use linear mixed models.

#### Dietary intake lists

Dietary intake lists will be summarized and evaluated by descriptive statistics. Relation between dietary intake and microbiome composition will be analyzed using a Pearson correlation coefficient.

### **10.3 Other study parameters**

Global hepatic mRNA expression baseline vs end of treatment (week 21). This analysis will be done by employing integrative multi-omics techniques such as mixOmics to analyze the changes in global hepatic mRNA expression in the liver and connect these changes to the changes in histology and in responders and non-responders.

### **10.4 Interim analysis (if applicable)**

No interim analysis will be performed as this is not feasible due to the methodology of the study. Nevertheless, a DSMB will be installed to ensure safety of the study participants.

## **11. ETHICAL CONSIDERATIONS**

### **11.1 Regulation statement**

This study will be conducted in accordance with the principles of the Declaration of Helsinki (latest version, October 2013) and in accordance with the regulations in the Medical Research Involving Human Subjects Act (WMO)

### **11.2 Recruitment and consent**

A study-specific subject information sheet and consent form for each part of the study will be prepared in accordance with the principles of GCP, the Declaration of Helsinki and all applicable laws and regulations. The information sheet will clearly explain the nature of the study, including the study objectives, the potential risks and benefits, and the procedures involved for the subject if he/she was to take part. For each individual volunteer, the investigator or designee will provide a detailed explanation of the study, both verbally and with the use of the written subject information sheet. The subject will receive enough time of reflection to consider participation. If the subject decides to participate in the study, the informed consent form must be signed and dated by the subject and the investigator (or designee who conducted the informed consent discussion). The original signed informed consent form must be stored in the trial master file at the trial unit. The date that informed consent was provided must be recorded in the subject's eCRF. A copy of the signed informed consent form and the information sheet must be given to the subject. The study-specific informed consent form must be signed before any protocol-related procedures are performed. The subject information sheet should be revised whenever important new information becomes available that may be relevant to the subject's consent. Any revised written information and consent form should receive the IEC's approval prior to use.

This study is performed in patients with NAFLD-related liver fibrosis and will be performed in the Amsterdam UMC. NAFLD-related fibrosis must be diagnosed with the gold standard, a liver biopsy. Individuals who recently were diagnosed with NAFLD-related fibrosis and had a fibrosis score  $\geq 2$  (i.e., perisinusoidal and portal/periportal fibrosis) based on a liver biopsy that was performed in the past six months are eligible for inclusion. No additional liver biopsy has to be performed. With the establishment of a dedicated NAFLD outpatient clinic, located at two sites in Amsterdam, in which we made a rapid start with inclusion of patients with NAFLD (biopsy proven) we aim to include as much possible patients that already underwent a liver biopsy.

In addition, patients will also be recruited through advertisement. However, we are well aware that performing liver biopsies is not without risk. Bearing in mind the invasive nature of a liver biopsy we designed an inclusion strategy in collaboration with the NLV to dramatically lower the change of performing a liver biopsy in individuals who do not have NAFLD-related fibrosis. The screening procedure is divided into three parts.

1. Assessment inclusion and exclusion criteria and measurement of non-invasive test for liver fibrosis using the Fibrosis-4 (FIB-4) index for liver fibrosis.

The (FIB-4) index will be calculated using the formula:  $\text{Age (years)} \times \text{AST U/l} / [\text{PLT}(109/\text{l}) \times \text{ALT}1/2 (\text{U/l})]$ . The negative predictive value for excluding patients with a fibrosis score  $<2$  is excellent. Those patients with FIB-4  $>1.5$  will undergo a non-invasive liver elastography.

2. Liver elastography (Fibroscan).

Liver elastography is a type of imaging test that checks the liver for fibrosis and is now considered to be the most promising noninvasive method for assessing liver fibrosis. The technique is fast, reproducible and does not cause discomfort or risks to the patient. The negative and positive predictive value for liver fibrosis is excellent. Patients that have a liver elastography score  $>7.5$  kilopascal will undergo a liver biopsy which is the last step in the screening procedure.

3. Ultrasound-guided liver biopsy

The liver biopsy is still the gold standard for diagnosis NAFLD and NAFLD-related liver fibrosis. The biopsy will be performed by either an experienced intervention radiologist from the AMC Department of Radiology or hepatologist from the AMC department of gastroenterology. The histopathological diagnosis of NAFLD and liver fibrosis will be assessed using the SAF (Steatosis, Activity, Fibrosis) scoring system. If the patient has NAFLD-related fibrosis with a fibrosis score  $\geq 2$ , the patient is eligible for inclusion.

### **11.3 Objection by minors or incapacitated subjects (if applicable)**

No minors or incapacitated subjects will be included.

### **11.4 Benefits and risks assessment, group relatedness**

NAFLD is estimated to affect approximately 25-30% of the world population and is now the leading cause of chronic liver disease globally<sup>1</sup>. NAFLD is a progressive liver disease and approximately 30% of individuals progress from simple steatosis to NASH, which can further progress to cirrhosis and hepatocellular carcinoma. In the Netherlands, it is estimated that 2.5 million people have NAFLD, and this number is thought to increase by 50% in the next 10 years driven by an increasing prevalence of obesity and T2DM, and an ageing population. Independent of other cardiometabolic diseases, cardiovascular disease (CVD) is the leading cause of death in individuals with NAFLD, followed by extrahepatic malignancies and liver-related complications. NAFLD results in sustained healthcare costs and economic losses, and reduced health-related quality of life.

It is now widely accepted that liver fibrosis is a result of liver injury secondary to NAFLD and is considered to be a major predictor for liver-related and overall mortality in individuals with NAFLD<sup>5</sup>. The process of fibrosis progression is not completely understood, and it can vary considerably from one individual to another. Several risk factors for fibrosis progression have been identified: age, hypertension, obesity and

T2DM. As of to date, no treatment is available that proved to be successful to target hepatic fibrosis. The only therapeutic options currently available therefore are the control of the concomitant metabolic diseases in addition to diet and lifestyle changes. Unfortunately, this inevitably will lead to polypharmacy and thereby decreases treatment adherence and increases the risk of adverse events and interactions with other drugs.

However, especially individuals at higher risk of disease progression who already have established hepatic fibrosis, pharmacologic treatment is required since diet and lifestyle is less successful to reverse fibrosis. In view of the strong preclinical data and first in human trials showing that fibrosis in adipose tissue decreased using dasatinib plus quercetin, this drug combination might be successful to target the major predictor of liver-related and cardiovascular outcome, hence hepatic fibrosis. In view of the expired patent for this drug, targeting senescence might be an innovative way to use generic (safe) medication to stop the continuous increase in the obesity-related burden on public health and economies.

### **11.5 Compensation for injury**

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO.

The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

### **11.6 Incentives (if applicable)**

Participants will be paid travel expenses or a second-class train ticket (including bus/metro ticket) when subjects hand over all documents (completed with a signature and appropriate date) related to the travel. A simple meal will be offered to the subject at the end of each study day. Participants will be paid €200,- for the totality of the study. In case of not completing the whole study, the amount will be adjusted accordingly. In case of a screening failure €50 and if not completing the study, €75.

## **12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION**

### **12.1 Handling and storage of data and documents**

Data will be collected on data collecting forms and will be entered after validation in a computer system for subsequent tabulation and statistical analysis. All research and medical data will be kept strictly confidential and registered under a unique study code. The key to this code will only be known to the principal- and coordinating investigators. Only the researchers that are involved in this study will be able to see the data and to identify a participant. All data will be kept at the AMC after the study has been completed, study material will be stored for a period of 15 years, except for biological samples, which will be stored for a period of maximum 5 years. By signing the informed consent, participants allow future analysis of stored material. Healthy and Youth Care Inspectorate and representatives of the Amsterdam UMC as sponsor of this study have access to the data at any time. This information will be included in the written informed consent.

### **12.2 Monitoring and Quality Assurance**

Monitoring will be done by an independent monitor provided by the CRU of the Amsterdam UMC, location AMC. Because the study was classified as **moderate risk**, monitoring will consist of an initiation visit and after each treatment cycle. The monitor will focus on the quality of data collection for the primary endpoint and patient safety.

A monitoring plan is currently developed by the monitor.

### **12.3 Amendments**

A 'substantial amendment' is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;
- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC and to the competent authority.

Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

### **12.4 Annual progress report**

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

### **12.5 Temporary halt and (prematurely) end of study report**

The sponsor will notify the accredited METC and the competent authority of the end of the study within a period of 90 days. The end of the study is defined as the last patient's last visit.

The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the sponsor will notify the accredited METC and the competent authority within 15 days, including the reasons for the premature termination. Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC and the Competent Authority.

### **12.6 Public disclosure and publication policy**

Publications will be open access and include anonymized data to the extent allowed by the new privacy legislation (AVG). The funding parties have no influence on the content or timing of the publication. The trial and protocol will be registered in a public trial registry before inclusion of the first patient. After the end of the study, raw data will be made available on request. The requests will be discussed by the principal investigator and project leader

## 13. STRUCTURED RISK ANALYSIS

### 13.1 Potential issues of concern

#### a. Level of knowledge about mechanism of action

Drugs that eliminate senescent cells are known as “senolytic(s)”. Senolytic agents were first discovered in 2015. Although already in 1995 it was discovered that senescent cells are resistant to apoptosis, it was later shown that senescent cells depend on SCAPs, which permit senescent cells to survive their own SASP. Using a combination of bioinformatic tools and *in vitro* RNA interference studies, it was verified that senescent cells rely on the SCAPs. Hence, SCAPs were identified as the Achilles heel of senescent cells. Since this discovery, considerable progress has been made in identifying small molecules, peptides and antibodies that selectively induce apoptosis in senescent cells. The combination of dasatinib, which is an EMA-approved tyrosine kinase inhibitor and the antioxidant quercetin, which is a flavonol present in many fruits and vegetables, successfully induce apoptosis in senescent cells *in vitro* and rodent models. According to *in vitro* data and later clinical data, a brief disruption of pro-survival pathways is adequate to suggest that senolytics could be administered intermittently, which reduced the risks of adverse effects compared to continuous treatment.

#### b. Previous exposure of human beings with the test product(s) and/or products with a similar biological mechanism

Worldwide hundreds of individuals have been enrolled in senolytic clinical trials using dasatinib plus quercetin for a variety of conditions, with no severe or serious side effects so far (Table 2, page 25-26). Nevertheless, it is important to note that dasatinib can have serious side effects. Side-effects of dasatinib include abdominal pain, vomiting, diarrhea, musculoskeletal pain, tiredness, and myelosuppression. Another side effect, which may occur after months of continuous use, include peripheral edema and pleural effusions. Importantly, it has been described that a twice daily dasatinib regimen, as well as a history of cardiac or autoimmune disease, are suspected as predisposing factors<sup>18,19</sup>. To minimize the risks, these predisposing factors were included into the exclusion criteria for the present RCT. In addition, pleural effusion is driven by a reactive oxygen species (ROS) dependent mechanism. Of interest, not only are these side effects reversible, but a recent study showed that the increased endothelial permeability, which drives the side-effects can be counteracted, using antioxidants such quercetin<sup>20</sup>. Hence, quercetin is not only a senolytic agent by itself, but the combination of dasatinib plus quercetin has a high potential to be effective and safe drug combination to selectively target age related diseases via an intermittent treatment strategy.

#### c. Can the primary or secondary mechanism be induced in animals and/or in ex-vivo human cell material?

In the past decade, an overwhelming amount of evidence has shown that targeting cellular senescence is potentially effective for a wide range of cardiometabolic diseases. First-

generation senolytics, such as dasatinib plus quercetin have been tested in several preclinical models of aging and diseases, including NAFLD, liver fibrosis, type 2 diabetes, and bone, heart, kidney, liver, lung, muscle and neurological disorders (Table 1, page 22-23). Whereas transplanting senescent cells decreases health span and lifespan in preclinical models, eliminating transplanted or endogenous senescent cells increases health span, thereby fulfilling Koch's postulates for causality

d. Selectivity of the mechanism to target tissue in animals and/or human beings

The combination of dasatinib and quercetin acts on multiple SCAP network nodes and thereby increases the potency towards clearing senescent cells. The combination of dasatinib and quercetin strongly reduces off-target apoptotic effects on non-senescent cell types, such as platelets and immune cells. This prevents 'panolytic' actions and thereby flattens the side-effect profile. Moreover, according to *in vitro* data, and later clinical data a brief disruption of pro-survival pathways is adequate to clear senescent cells<sup>8,9</sup>. Because senescent cells take weeks to reaccumulate, senolytics can be administered intermittently, which reduces the risk of adverse effects compared to continuous treatment. This was already shown in multiple animal studies as shown in table 1, page 22-23), but also in the two recent clinical trials<sup>34,36</sup>.

e. Analysis of potential effect

The effect of the study is evaluated by using a liver biopsy. Although a liver biopsy is an invasive procedure, it remains the gold standard for the diagnosis of NAFLD and is therefore required for the interpretation of the results. We, however, are well aware of the invasive nature of the biopsies. Therefore, we aim to include as many individuals via our outpatient NAFLD clinic. The individuals already underwent a liver biopsy. If the biopsy was performed within six months, only one additional biopsy is necessary for this trial. Liver biopsies are performed after informed consent, via our AMC intervention radiology department or gastroenterology department for an ultrasound guided liver biopsy to assess NAFLD histology. As an experienced hepatologist or interventional radiologist will perform the liver biopsy ultrasound- guided, the risk (comprising mostly bleeding from the biopsy sites) of complications will be very low (<0.1%). Moreover, local hemostasis after the procedure will be observed and bleeding disorders are an exclusion criterion.

f. Pharmacokinetic considerations

Pharmacokinetics of dasatinib are well described and investigated. One of the most important things is that in contrast to other tyrosine kinase inhibitors, dasatinib usage is not associated with an increased risk of hepatotoxicity<sup>16</sup>. Also, in the first human trials and extensive data from pre-clinical models, it was shown that despite an intermittent treatment with dasatinib plus quercetin is clinically effective despite the short elimination half-life of dasatinib and also quercetin (5-6 hours),

g. Study population

This study will consist of individuals with NAFLD-related liver fibrosis. As mentioned

previously, there is now substantive and consistent evidence that NAFLD-related fibrosis, is the strongest predictor of future all-cause mortality and morbidity, with a 5–12-fold increase in Relative Risk of death and liver-related events, including liver failure, transplantation, and liver cancer. NAFLD results in sustained healthcare costs and economic losses, and reduced health-related quality of life. Indeed, according to real world data, the mean total annual per patient cost of NAFLD was estimated at €2,763, €4,917, and €5,509 for the direct medical, direct non-medical, and indirect cost categories, respectively<sup>63</sup>. Therefore, targeting NAFLD and especially NAFLD-related fibrosis with the aim to prevent disease progression is an incremental component of patient management strategies.

#### h. Interaction with other products

There are interactions with other products. We have extensively described the possible interactions in section 5.2

#### i. Predictability of effect

According to studies in mice and humans, the combination of dasatinib plus quercetin is safe and successful and effects lasts for approximately 3-4 weeks after cessation of the treatment.

For dasatinib, there are several side effects and the groups that are more vulnerable are well described. Characteristics of these patients are included in the exclusion criteria. Examples of most severe side effects and in which patients group the side effects more frequently occur are described below.

#### Myelosuppression:

The risk of myelosuppression is dose dependent. Myelosuppression is more frequent in patients with advanced phase of CML or Ph+ ALL than in CP CML. In subjects not affected with ALL or CML, myelosuppression did not occur.

#### Fluid retention:

Risk factors include older age, fluid retention at baseline, prior imatinib treatment, and renal impairment.

While the safety profile of dasatinib in the elderly population was generally like that in the younger population, the incidence of pleural effusion increases with age. For example, patients aged 65 years and older are more likely to experience fluid retention events and should be monitored closely.

#### Bleeding risk:

Patients with leukaemia, severe thrombocytopaenia, coagulation disorder, cardiovascular disorders, and patients who take medicinal products that inhibit platelet function or anticoagulants.

#### QT prolongation:

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Patients with hypokalaemia or hypomagnesaemia, patients with congenital long QT syndrome, patients taking anti-arrhythmic medicinal products or other medicinal products which lead to QT prolongation, and cumulative high dose anthracycline therapy.

Hepatotoxicity:

No risk factors have been identified for subjects developing hepatic AEs with dasatinib.

j. Can effects be managed?

One of the most important precautions we made was including risk factors for side effects into the exclusion criteria. If side effects occur, according to the SPC of dasatinib discontinuation of the medication is in most of the cases sufficient for reversal.

## **13.2 Synthesis**

It is now widely accepted that liver fibrosis is a result of liver injury secondary to NAFLD and is considered to be a major predictor for liver-related and overall mortality in individuals with NAFLD<sup>5</sup>. As of to date, no treatment is available that proved to be successful to target hepatic fibrosis. The only therapeutic options currently available therefore are the control of the concomitant metabolic diseases in addition to diet and lifestyle changes. In view of the strong preclinical data and first in human trials showing that fibrosis in adipose tissue decreased using dasatinib plus quercetin, this drug combination might be successful to target the major predictor of liver related and cardiovascular outcome, hence hepatic fibrosis.

With the help of the NLV we have weighted the burden and associated risks with participation to the potential benefit. Nevertheless, the study can be considered extensive and invasive for the participants due to following procedures:

Liver biopsy: Study participants must undergo a liver biopsy during the course of this study because a Fibroscan, MRI or ultrasound, although non-invasive are inferior for the accurate diagnosis and are therefore not used in clinical trials for primary endpoints<sup>15</sup>. Although a liver biopsy is an invasive procedure, it remains the gold standard for the diagnosis of NAFLD and is therefore required for the interpretation of the results. Moreover, given the fact that the individuals are recruited via our outpatient NAFLD clinic, oftentimes a biopsy of the liver has already been performed. Therefore, only one additional biopsy is necessary for this trial. Liver biopsies are performed after informed consent, via our AMC intervention radiology department or gastroenterology department for an ultrasound guided liver biopsy to assess NAFLD histology. As an experienced hepatologist or interventional radiologist will perform the liver biopsy ultrasound- guided, the risk (comprising mostly bleeding from the biopsy sites) of complications will be very low (<0.1%). Moreover, local hemostasis after the procedure will be observed and bleeding disorders are an exclusion criterion.

Safety and tolerability: A possible pitfall is the safety and tolerability of the senolytic drug. However, intermittent dasatinib plus quercetin appears to have an exceptionally good safety profile<sup>13</sup>. Accumulative over hundreds of individuals are enrolled worldwide in senolytic clinical trials using dasatinib plus quercetin for a variety of conditions, with no severe or serious side effects so far. By itself, dasatinib has been approved as a prescription drug in the EU for various cancers and also for fibrotic diseases (scleroderma, progressive systemic sclerosis), usually at doses of 100 mg/day for life. Of interest, in contrast to other tyrosine kinase inhibitors, dasatinib usage is not associated with an increased risk of hepatotoxicity<sup>16</sup>. Recently the suggestion that dasatinib is associated with improved glucose metabolism and decreased need for glucose lowering medication was further substantiated<sup>17</sup>. Side-effects of dasatinib include abdominal pain, vomiting, diarrhea, musculoskeletal pain, tiredness, and myelosuppression. Another side effect, which may occur after months of continuous use, include peripheral edema and pleural effusions. Importantly, it has been described that a twice daily dasatinib regimen, as well as a history of cardiac or autoimmune disease, are suspected as predisposing factors<sup>18,19</sup>. To minimize the risks, these predisposing factors were included into the exclusion criteria for the present RCT. In addition, pleural effusion is driven by a ROS dependent mechanism. Of interest, not only are these side effects reversible, but a recent study showed that the increased endothelial permeability, which drives the side-effects can be counteracted, using antioxidants such quercetin<sup>20</sup>. Hence, quercetin is not only a senolytic agent by itself, but the combination of dasatinib plus quercetin has a high potential to be effective and safe drug combination to selectively target age related diseases via an intermittent treatment strategy.

Treatment adherence: In the past decades, extensive research has given important insight into the many complex factors that interact to affect how and why individuals take prescribed drugs. The focus has been on the consumers factors that relate to non-compliance. However, there is also growing awareness that factors beyond consumers' control can affect adherence. It is now widely accepted that communication in its entirety is critical, and there is increasingly a view that interventions to improve adherence should focus not just on consumers but on the wider patient context and healthcare system. An emerging theme among recent research is an emphasis on adopting patient-centered care and shared decision-making principles in order to achieve better adherence, together with attention to barriers that may be targets for interventions.

With the input of the NLV, we have a fundamental basis that our proposed intermittent treatment strategy is attractive and could increase compliance in similar way the once weekly GLP-1 injection or vitamin D supplementation treatment strategy. Both patient associations mentioned that regular contact with patients should be implemented in the study to remind the individuals to take their medication to increase the adherence. Therefore, throughout the trial, the investigator will remind the subjects to follow the trial procedures and requirements to ensure the individuals compliance. If an individual is found to be non-compliant the investigator will remind the individual of the importance of following the instructions given including taking the trial products as prescribed. Treatment compliance of trial product will be assessed by asking individual about missed doses and

monitoring of dosing diaries. Information about compliance and missed doses should be described in the individual's medical record.

All in all, this work is important considering the fact that there is currently no registered treatment for NAFLD. Moreover, patients with NAFLD often have cardiometabolic comorbidity, which can lead to polypharmacy. Senolytics can be administrated intermittently/pulsatile, which reduces the risks of adverse effects and drug interactions. In view of the expired patent of dasatinib, targeting senescence might be a safe and innovative way to use generic and safe medication to reserve progressive stages of NAFLD.

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| <b>Fibrosis gene signature</b> | <b>SenMayo signature</b> | <b>SHGS signature</b> |
|--------------------------------|--------------------------|-----------------------|
| COL1A1                         | <i>ACVR1B</i>            | LIF                   |
| COL1A2                         | <i>ANG</i>               | ITGAV                 |
| COL3A1                         | <i>ANGPT1</i>            | OSBPL3                |
| COL5A1                         | <i>ANGPTL4</i>           | IFNGR1                |
| COL6A1                         | <i>AREG</i>              | THBS1                 |
| COL6A2                         | <i>AXL</i>               | NPDC1                 |
| COL6A3                         | <i>BEX3</i>              | PLCD3                 |
| FN1                            | <i>BMP2</i>              | CADM1                 |
| VCAN                           | <i>BMP6</i>              | SEMA3E                |
| MMP2                           | <i>C3</i>                | SPP1                  |
| MMP9                           | <i>CCL1</i>              | DUSP4                 |
| MMP14                          | <i>CCL13</i>             | SH3BGRL3              |
| TIMP1                          | <i>CCL16</i>             | CXCL9                 |
| TIMP2                          | <i>CCL2</i>              | ITGA3                 |
| LOX                            | <i>CCL20</i>             | TAGLN2                |
| LOXL1                          | <i>CCL24</i>             | DNAJC10               |
| LOXL2                          | <i>CCL26</i>             | AREG                  |
| TGFB1                          | <i>CCL3</i>              | NBL1                  |
| TGFB2                          | <i>CCL3L1</i>            | LGALS3                |
| TGFB3                          | <i>CCL4</i>              | PPFIBP1               |
| PDGFRA                         | <i>CCL5</i>              | GPRC5A                |
| PDGFRB                         | <i>CCL7</i>              | QPCT                  |
| CCN2                           | <i>CCL8</i>              | IER3                  |
| ACTA2                          | <i>CD55</i>              | EPHA2                 |
| DES                            | <i>CD9</i>               | HBEGF                 |
| VIM                            | <i>CSF1</i>              | FGD3                  |
| SERPINE1                       | <i>CSF2</i>              | CPE                   |
|                                | <i>CSF2RB</i>            | EZR                   |
|                                | <i>CST4</i>              | ANKRD1                |
|                                | <i>CTNNB1</i>            | ITGA2                 |
|                                | <i>CTSB</i>              | GALNT1                |
|                                | <i>CXCL1</i>             | GBP2                  |
|                                | <i>CXCL10</i>            | EMP1                  |
|                                | <i>CXCL12</i>            | TPM1                  |
|                                | <i>CXCL16</i>            | GLS                   |
|                                | <i>CXCL2</i>             | ANGPTL4               |
|                                | <i>CXCL3</i>             | CAPN2                 |
|                                | <i>CXCL8</i>             | IGDCC4                |
|                                | <i>CXCR2</i>             | S100A6                |
|                                | <i>DKK1</i>              | SYNJ2                 |
|                                | <i>EDN1</i>              | PCDH7                 |
|                                | <i>EGF</i>               | SLC4A11               |
|                                | <i>EGFR</i>              | SNTB2                 |
|                                | <i>EREG</i>              | LTB                   |

|  |               |         |
|--|---------------|---------|
|  | <i>ESM1</i>   | MYOF    |
|  | <i>ETS2</i>   | CXCL5   |
|  | <i>FAS</i>    | CBARP   |
|  | <i>FGF1</i>   | PDP1    |
|  | <i>FGF2</i>   | PALMD   |
|  | <i>FGF7</i>   | FOSL1   |
|  | <i>GDF15</i>  | FADS3   |
|  | <i>GEM</i>    | PAWR    |
|  | <i>GMFG</i>   | THSD4   |
|  | <i>HGF</i>    | INPP4B  |
|  | <i>HMGB1</i>  | TANC1   |
|  | <i>ICAM1</i>  | TRIM35  |
|  | <i>ICAM3</i>  | TINAGL1 |
|  | <i>IGF1</i>   | MTCL1   |
|  | <i>IGFBP1</i> | ELOVL7  |
|  | <i>IGFBP2</i> | CXCL3   |
|  | <i>IGFBP3</i> | IGFBP3  |
|  | <i>IGFBP4</i> | JAG1    |
|  | <i>IGFBP5</i> | IQGAP1  |
|  | <i>IGFBP6</i> | CD9     |
|  | <i>IGFBP7</i> | CDKN2B  |
|  | <i>IL10</i>   | FBLIM1  |
|  | <i>IL13</i>   | CCND1   |
|  | <i>IL15</i>   | KIF5C   |
|  | <i>IL18</i>   | ANXA3   |
|  | <i>IL1A</i>   | FLNA    |
|  | <i>IL1B</i>   | RAB31   |
|  | <i>IL2</i>    | ITPR3   |
|  | <i>IL32</i>   | ENC1    |
|  | <i>IL6</i>    | TSPAN2  |
|  | <i>IL6ST</i>  | SRXN1   |
|  | <i>IL7</i>    | MYL12A  |
|  | <i>INHA</i>   | HKDC1   |
|  | <i>IQGAP2</i> | CDH17   |
|  | <i>ITGA2</i>  | ABHD2   |
|  | <i>ITPKA</i>  | KRT18   |
|  | <i>JUN</i>    | FHL2    |
|  | <i>KITLG</i>  | SYTL5   |
|  | <i>LCP1</i>   | BMP8B   |
|  | <i>MIF</i>    | VIM     |
|  | <i>MMP1</i>   | CD44    |
|  | <i>MMP10</i>  | ARL4C   |
|  | <i>MMP12</i>  | DDIT4L  |
|  | <i>MMP13</i>  | RHBDF1  |
|  | <i>MMP14</i>  | SLC30A4 |

|  |           |           |
|--|-----------|-----------|
|  | MMP2      | TNFRSF12A |
|  | MMP3      | PANX1     |
|  | MMP9      | PAM       |
|  | NAP1L4    | SERINC2   |
|  | NRG1      | SLC48A1   |
|  | PAPPA     | CD151     |
|  | PECAM1    | TGFBR2    |
|  | PGF       | SLPI      |
|  | PIGF      | WDR1      |
|  | PLAT      | RAP2B     |
|  | PLAU      | ASAH1     |
|  | PLAUR     |           |
|  | PTBP1     |           |
|  | PTGER2    |           |
|  | PTGES     |           |
|  | RPS6KA5   |           |
|  | SCAMP4    |           |
|  | SELPLG    |           |
|  | SEMA3F    |           |
|  | SERPINB4  |           |
|  | SERPINE1  |           |
|  | SERPINE2  |           |
|  | SPP1      |           |
|  | SPX       |           |
|  | TIMP2     |           |
|  | TNF       |           |
|  | TNFRSF10C |           |
|  | TNFRSF11B |           |
|  | TNFRSF1A  |           |
|  | TNFRSF1B  |           |
|  | TUBGCP2   |           |
|  | VEGFA     |           |
|  | VEGFC     |           |
|  | VEGF      |           |
|  | WNT16     |           |
|  | WNT2      |           |

| Group     | Visit | Cell_count |
|-----------|-------|------------|
| Placebo   | V2    | 11650      |
| Senolytic | V2    | 26743      |
| Placebo   | V5    | 19721      |
| Senolytic | V5    | 31607      |

| Celltype      | Cell_count |
|---------------|------------|
| Hepatocytes   | 66950      |
| Endothelial   | 7340       |
| Myeloid       | 3699       |
| B and T cells | 2945       |
| HSCs          | 5494       |
| Cholangiocyte | 3293       |

| Senolytics group | logFC        | AveExpr      | t            | P.Value     | adj.P.Val   | B        |
|------------------|--------------|--------------|--------------|-------------|-------------|----------|
| HSCs             | -0,303074618 | -2,774950608 | -1,129725456 | 0,270851986 | 0,823846218 | -4,58489 |
| Hepatocytes      | 0,1913869    | 1,063184166  | 0,676516502  | 0,505817698 | 0,823846218 | -4,62307 |
| B and T cells    | -0,254940784 | -3,676113939 | -0,668640729 | 0,510733367 | 0,823846218 | -4,62358 |
| Endothelial      | -0,161195719 | -2,480730271 | -0,608353211 | 0,549230812 | 0,823846218 | -4,62732 |
| Cholangiocytes   | 0,077659589  | -3,223937816 | 0,219761365  | 0,828098827 | 0,946484921 | -4,64319 |
| Myeloid          | 0,023693318  | -3,289588898 | 0,067897905  | 0,946484921 | 0,946484921 | -4,64538 |

| Placebo group  | logFC        | AveExpr      | t            | P.Value     | adj.P.Val   | B        |
|----------------|--------------|--------------|--------------|-------------|-------------|----------|
| Cholangiocytes | 1,466034464  | -3,747390277 | 5,417412589  | 1,44E-06    | 8,62E-06    | 7,771844 |
| B and T cells  | 1,282333827  | -4,213087192 | 4,738586701  | 1,61E-05    | 4,82E-05    | 4,378928 |
| Hepatocytes    | -0,618985242 | 1,542364947  | -2,287325791 | 0,026120685 | 0,052241369 | -4,09694 |
| HSCs           | 0,546564916  | -3,017387061 | 2,019712174  | 0,048387237 | 0,072580855 | -4,6642  |
| Myeloid        | 0,173790233  | -3,666622995 | 0,642204136  | 0,523457813 | 0,590637048 | -6,46879 |
| Endothelial    | 0,146440575  | -2,868489234 | 0,541139402  | 0,590637048 | 0,590637048 | -6,52765 |