DRCR Retina Network

Diabetic Retinopathy and Changes in Lipid Metabolism (Protocol AFA)

Version 3.0

06NOV2025

By signing below, I acknowledge my approval of this protocol.

JCHR Protocol Director

Name: Cynthia Stockdale, MSPH

Signature/Date: Cynthia Stockdale box SIGN 4QXV65V1-4K2RVW87
Dec 3, 2025

VERSION HISTORY

The following table lists effective versions of the protocol:

VERSION NUMBER	AUTHOR	APPROVER	EFFECTIVE DATE	REVISION DESCRIPTION
1.0	Crystal Franklin	Cynthia Stockdale	Not Implemented	Initial IRB Submission (not implemented)
1.1	Crystal Franklin	Cynthia Stockdale	Not Implemented	Healthy volunteer will not complete FA testing; clarified genetic testing
2.0	Crystal Franklin	Cynthia Stockdale	16AUG2024	Remove CI-DME as an exclusion criterion, clarification for AF Cohort 2 consent/analysis, prior procedures to treat DR, FA transit eye procedures, and addition of windows for screening tests
3.0	Sandra Galusic	Cynthia Stockdale	03DEC2025	Update to analysis group 3 to reflect recruitment feasibility, reduced sample size and added language for ending recruitment early Revisions throughout for clarity namely how enrollment is handled based on site comments to date, inclusion/exclusion criteria, genetics consent and allowable equipment for study testing; update of Fred Hutch RCB name to BPB

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Chapter 1: Introduction

1.1 Background

Diabetic retinopathy (DR) and associated changes are considered a leading cause of blindness in the western world.[1] Long-term diabetes status for ≥10 years leads to DR in up to 80% of affected patients.[2] Different factors, including clinical, genetic, epigenetic, biochemical, and molecular factors, have been shown to contribute to the risk of diabetic complications. Amongst these, a specific class of sphingolipids, the deoxysphingolipids, have been demonstrated to be elevated in both type 1 and 2 diabetes[3], and to be associated with an increased risk of developing diabetic polyneuropathy.[4]

Deoxysphingolipids are increased in patients with diabetes mellitus type 2[3 7] as well as in patients with metabolic syndrome.[8] In type 1 diabetes, elevated circulating levels of deoxysphingolipids have been shown to be associated with an increased risk of diabetic polyneuropathy.[4] Furthermore, a correlation of retinal disease severity and circulating deoxysphingolipid levels has recently been shown in another retinal disease, macular telangiectasia type 2 (MacTel).[9] Different factors have been displayed to be associated with the development of diabetic retinopathy, including the duration of diabetes[10], HbA1c levels[11] and microalbuminuria.[12] So far, associations between sphingolipid metabolism and the risk for developing diabetic retinopathy as well as the severity of diabetic retinopathy have not been examined.

1.2 Rationale

A Randomized Clinical Trial Evaluating Fenofibrate for Prevention of Diabetic Retinopathy Worsening (Protocol AF) is a randomized clinical trial that will evaluate the effect of fenofibrate compared with placebo for prevention of DR worsening through 6 years of follow-up in eyes with mild to moderately severe non-proliferative DR (NPDR) and no CI-DME at baseline. While the primary objective is to determine if fenofibrate is effective at preventing DR worsening, an additional goal is to collect information on potential predictive biomarkers via blood sampling over the course of DR progression.

To help evaluate potential predictive biomarkers across varying levels of DR severity, Protocol AFA will recruit populations with DR severity differing from the Protocol AF cohort. The study visit procedures for participants enrolled in Protocol AFA will mirror those from Protocol AF at the Screening visit, with the exception that ancillary ocular procedures (OCTA and Contrast Sensitivity) typically performed at randomization will also be performed. As detailed in the Statistical Considerations chapter below, data from Protocol AF participants (who consented to allow the sharing of de-identified data and de-identified serum sample use for future research) and also consented to Protocol-GENES will be pooled with the Protocol AFA participants to evaluate changes across the DR severity spectrum.

Protocol AFA is a collaborative effort between the Jaeb Center for Health Research and the Lowy Medical Research Institute (LMRI), Ltd. LMRI will be performing the metabolite analyses with the de-identified serum samples and also will be submitting a request to the JCHR IRB to obtain samples from the Protocol-GENES for genetic analyses under this protocol for the participants contributing data from the Protocol AF cohort. LMRI will not be saving (banking)

the genetic samples for future research and will be performing all sample testing at facility(ies) within the United States in accordance with the terms of written agreement(s). This protocol serves to describe the new cohort being enrolled for AFA as well as describe the planned analyses from the cumulative data and samples, as specified, for this initiative.

1.3 Objectives

1. Determine if circulating metabolites, including sphingolipids, are associated with severity of DR in participants with diabetes mellitus type 2.

2. Identify metabolic risk and protective factors that are associated with the development and progression of DR.

1.4 Protocol Synopsis

Protocol AFA will enroll approximately 200 participants whose data will be pooled with approximately 200 randomized Protocol AF participants to compare metabolic changes associated with DR severity level. To conduct the analyses, participants from Protocols AF and AFA with diabetes will be placed into Groups 1 through 4, based on level of DR severity, as seen in Table 1. The new AFA cohort will consist of approximately 160 participants with diabetes mellitus type 2 and DR severity levels falling under the AF eligibility cut-off (No DR to Mild, Group 1) or above the cut-off, Group 4 (PDR, Group 4), with 20 additional participants having Severe NPDR (Group 3) as well as 20 participants without diabetes as part of a non-diabetic control group (Group 5).

If both eyes are not of the same DR severity, the participant's worse eye will be decisive for the final classification into each of the designated groups. Since classification is based on Reading Center assessment of DR severity level, it is possible for cohorts to be overenrolled. Additionally, some AFA-enrolled participants may fall in Group 2 and will be included in the analysis if AF-only cohort target goal is not otherwise met. Eyes enrolled into Group 3 will be based on reading center grading only, consisting of AF screen fail patients and those AFA-enrolled eyes intended for other cohorts.

Table 1. Description of Groups for Analyses from Protocols AF & AFA

Analysis Group Number	Group Description Participants with Type 2 Diabetes, and worse eye:	Target Number of Participants
AFA Group 1	Absent of DR, or microaneurysms only (Diabetes ≥15 yrs) **(DR severity level 10 to 20)**	**80**
Protocol AF Cohort, Group 2	Mild to moderately severe DR **(DR severity level 35 to 47)**	**200**
AFA Group 3	Severe non-proliferative DR **(DR severity level 53)**	**20**
AFA Group 4	Proliferative DR **(DR severity level 61 to 81)**	**80**
AFA Group 5	**Absent of any diagnosis of diabetes and absent evidence of retinal disease as determined by color fundus photography and OCT imaging**	**20**
Total Number of Participants in the Analysis Pool (AF & AFA)		**400**

Prior to completing any procedures or collecting any data for this research, informed consent for either Protocols AF and GENES, or Protocol AFA will be obtained (see section 2.2). Participants who screen fail for participation in Protocol AF may still be eligible for Protocol AFA, with re-screening only necessary if sample collection and/or any testing procedures listed below was not completed.

Participants will undergo a screening evaluation to determine eligibility for the AFA cohort. Qualified participants must meet all eligibility criteria. All procedures conducted during the Protocol AF screening visit will likewise be conducted for Protocol AFA participants, including: medical history, visual acuity, OCT, fundus photography, fluorescein angiography (FA), physical exam, fasting blood samples, and urine sample. Non-diabetic patients (Group 5) will not undergo fluorescein angiography. Participants from the Protocol AF cohort must also have provided a genetics sample under DRCR Protocol-GENES to be included in the analyses for Protocol AFA. The study team will follow the DRCR Genetics Project Sample Requests Policy, including the submission of a Secondary Research of Stored Biospecimens xForm, under Protocol-GENES, to the IRB for review and approval. Once approved, the genetic samples from Protocol-GENES can be provided to LMRI to conduct SNP typing on all participants using aliquot(s) from the provided genetics sample.

1.5 Potential Risks and Benefits of the Study

1.5.1 Known Potential Risks Related to Common Procedures

Many of the procedures in this study are part of daily ophthalmologic practice for people with diabetic retinopathy and pose few known risks. Dilating eye drops will be used as part of the exam. There is a small risk of inducing a narrow-angle glaucoma attack from the pupil dilation. However, all participants will have had prior pupil dilation usually on multiple occasions and therefore the risk is extremely small. For fluorescein angiography, there is a small risk of discomfort or phlebitis at the site of the injection. Lightheadedness or nausea after dye injection may occur and is usually transient and resolves after a few minutes without further intervention. An allergic reaction to the dye used to do fluorescein angiography imaging is rare. A rash or pruritus (itching) can develop, but true anaphylactic reactions are very rare. Fundus photographs, OCT and OCTA have bright lights associated with the camera flashes, which can be uncomfortable for study participants, but these carry no known risk to the eye or vision. Possible risks from blood draws include the following: bruising, arm discomfort, clotting, excess bleeding, infection, or fainting. Please note that although these are possible risks, they are unlikely.

Non-diabetic patients (Table 1, AFA Group 5) will not undergo fluorescein angiography but will do all other procedures.

1.5.2 Risks Related to Confidentiality

The risk of disclosure of protected health information is very small. Efforts are taken to assure that this does not occur, in compliance with HIPAA.

1.5.3 Known Potential Benefits

There will be no direct benefit to the participant from participating in this study. The participant's involvement may further knowledge about the relationship between metabolic levels and DR.

1.6 Risk Assessment

The protocol risk assessment for this non-intervention ancillary study has been categorized as no greater than minimal risk.

1.7 General Considerations

The study is being conducted in compliance with the policies described in the study policies document, with the ethical principles that have their origin in the Declaration of Helsinki, with the protocol described herein, and with the standards of Good Clinical Practice (GCP).

When feasible, data will be directly collected in electronic case report forms, which will be considered the source data.

Chapter 2: Study Enrollment and Screening

2.1 Participant Recruitment and Enrollment

A minimum of 400 participants are expected to be included in the analyses. Recruitment and enrollment for the Protocol AFA cohort will run concurrent with Protocol AF at approximately 80 clinical centers in the United States and Canada.

Participants who enroll in Protocol AF may contribute data to the analyses in accordance with their informed consent(s) from Protocol AF and Protocol GENES. In addition, there may be participants identified as ineligible for Protocol AF during routine-care examinations who may consent to be part of the AFA cohort.

2.2 Informed Consent

Potential eligibility may be assessed as part of a routine-care examination. Before completing any study procedures or collecting any data for the study, written informed consent will be obtained.

(1) Participants potentially meeting eligibility for Protocol AF may complete the informed consent forms (ICFs) for Protocols AF and GENES and proceed with screening thereafter. This group may contribute data to the AFA analyses in accordance with their informed consent(s) from Protocol AF and Protocol GENES.

(2) Participants not meeting the inclusion criteria of Protocol AF, or who cannot commit to a longitudinal study, may take part by being consented using the ICF for the Protocol AFA cohort, which is designed specifically for participants who are not taking part in Protocols AF and GENES respectively.

(3) Participants without diabetes may take part by being consented using the ICF for the Protocol AFA cohort. These participants should be in generally good health with no history of diabetes. They should have no eye diseases as evidenced by OCT or color fundus photography. Unlike the population with a history of diabetes, these participants will not complete the fluorescein angiography procedure.

The study protocol will be discussed with the potential study participant by study staff. The potential study participant will be given the Informed Consent Form to read. Potential study participants will be encouraged to discuss the study with family members and their personal physicians(s) before deciding whether to participate in the study.

As part of the informed consent process, each participant will be asked to provide authorization for release of personal information. The investigator, or his or her designee, will review the study-specific information that will be collected and to whom that information will be disclosed. After speaking with the participant, questions will be answered about the details regarding authorization.

A participant is considered enrolled when the applicable informed consent forms have been signed.

2.3 AFA Population

Approximately 200 AFA participants will be recruited from clinical centers already certified to recruit for Protocols AF and GENES. Data from approximately 200 randomized Protocol AF participants that have also consented for Protocol-GENES will be pooled with the AFA-only cohort for the analyses.

Participants diagnosed with diabetes mellitus type 2 will fall into Groups 1, 2, 3, or 4:

- Group 1 – DR Severity level 10 to 20 (n=80): participants in Group 1 must have been diagnosed with DM for 15 years or longer.
- Group 2 – DR Severity 35-47 (n=200, most of which will be randomized in Protocol AF)
- Group 3 – DR Severity level 53 (n=20): participants in Group 3 will qualify based on reading center grading only from eyes enrolled in the other groups.
- Group 4 – DR severity level 61 to 81 (n=80)

The participant's worse eye based on reading center grading will be decisive for the final classification of a patient in the above-mentioned categories. Participants in these groups may include a participant that screen failed for Protocol AF due to DR severity level or may include a participant that was known to be ineligible for Protocol AF and specifically consented as part of the AFA-only cohort. Given the rapid rate of progression in level 53 eyes and difficulty in capturing key severe NPDR features on imaging, eyes enrolled into Group 3 will be based on reading center grading only, consisting of AF screen fail patients and those AFA-enrolled eyes intended for other cohorts. Approximately 20 eyes are expected to be included in this group.

20 participants of generally good health, without any diabetes-related conditions and without any evidence of retinal diseases will be enrolled into Group 5 as part of a small non-diabetic control group (AFA only cohort). These participants will complete all AFA procedures, with the exception of the digital fluorescein angiography.

2.4 AFA-only Cohort - Participant Inclusion Criteria

Individual-level Criteria

To be eligible, the following inclusion criteria must be met:

1. Age ≥ 18 and < 80 years
   - *Individuals <18 years old are not being included because DR is so rare in this age group that the diagnosis of NPDR may be questionable. Individuals ≥80 years old are excluded to match the Protocol AF lead study age criterion.*
2. Diagnosis of type 2 diabetes mellitus, falling into one of the following categories:
   - For Group 1 participants with DR Severity level 10 to 20 the patient must have been diagnosed ≥15 years ago. If diagnosis of diabetes was <15 years, the participant is not eligible.
   - Any amount of time if DR Severity level ≥ 35 (Groups 2-4)
     - Any one of the following will be considered to be sufficient evidence that diabetes is present:

- *Current regular use of insulin for the treatment of diabetes.*
- *Current regular use of oral anti-hyperglycemia agents for the treatment of diabetes.*
- *Documented diabetes by American Diabetes Association and/or the World Health Organization criteria.*

OR

- Group 5 participants (non-diabetic control group must have generally good health in the opinion of the investigator, no history of documented diabetes or diabetes-related condition, HbA1c levels < 5.7 as confirmed by the central laboratory, must meet none of the exclusion criteria listed below, and must have no evidence of retinal diseases on color fundus photos or OCT.

3. Media clarity, pupillary dilation, and study participant cooperation sufficient to obtain adequate fundus photographs, FA, and OCT.
   - *Investigator must verify accuracy of OCT scan by ensuring it is centered and of adequate quality (including segmentation line placement)*
4. Able and willing to provide informed consent and participate (i.e., proficient in written and spoken English, Spanish or other IRB-approved consent language; has the capacity to consent for one's own self).
5. Able and willing to provide genetics sample.

2.5 Participant Exclusion Criteria

1. A condition that, in the opinion of the investigator, would preclude participation in the study such as unable or unwilling to complete all study procedures.
2. Participation in an investigational trial that involved treatment within last 30 days, with the exception of the parent Protocol AF.
3. Known allergy to fluorescein dye.
4. Participant has any retinal condition that complicates the diagnosis and grading of DR (e.g., previous therapies (vitreo-retinal surgery, photodynamic therapy)) or participant shows signs of retinal diseases other than DR, such nAMD or MacTel.
   - *Prior procedures to treat DR (e.g., intraocular anti-VEGF, vitrectomy) are not exclusionary so long as they do not interfere with the evaluation and grading of DR; participants with history of PRP in either eye are excluded since the study does not include DR severity of 60.*
5. Participant is currently taking or has been taking a PPAR-agonist within the last 90 days.
6. Participant is currently taking a serine or glycine supplement or has been taking a serine or glycine supplement within the last 60 days.
7. Employed in an involved research department or ophthalmologic practice participating in this research.
8. People who are currently pregnant and/or lactating.

2.6 Screening Procedures

After informed consent has been signed, a potential participant will be evaluated for study eligibility through the elicitation of a medical history and performance of an ocular examination by study personnel to screen for exclusionary conditions.

2.6.1 Data Collection and Testing

All testing does not need to be completed on the same day provided it is within the windows specified in the visit procedures. The visit should be scheduled early in the day to accommodate fasting requirements for blood samples.

Chapter 3: Study Visit Procedures

3.1.1 Visit Procedures

The following procedures are needed from participants screened specifically for the AFA cohort to confirm eligibility:

- If a procedure has been performed using the study technique and by study certified personnel as part of usual care, then it does not need to be repeated specifically for the study if it was performed within the defined time windows specified below.
- The testing procedures are detailed in the DRCR Retina Network procedures manuals.
- Visit procedures will last approximately 4 hours.

1. Self-reported demographics (date of birth, sex, race and ethnicity)
2. Medical history (pre-existing medical conditions, concomitant medications, as well as ocular diseases, surgeries, and treatment)
   - ➢ Medical history will be obtained by medical charts if available at the enrolling site; otherwise, it will be self-reported
3. Protocol refraction followed by electronic-ETDRS visual acuity testing using the Electronic Visual Acuity Tester or ETDRS charts in both eyes *(within prior 8 days of ocular examination).*
   - *Snellen charts are not acceptable for VA testing*
4. Digital fundus photographs on both eyes using the widest approach available (e.g., Optos ultra-widefield imaging device, if available) *(within prior 8 days of ocular examination).*
5. Spectral-domain OCT using Heidelberg Spectralis in both eyes *(within prior 8 days of ocular examination).*
   - ➢ All OCTs at screening will be sent to a reading center for manual grading
   - ➢ Includes additional optic nerve head scan to obtain Retinal Nerve Fiber Layer (RNFL) thickness data.
6. Digital FA – only for participants with diabetes
   - ➢ FA will **NOT** be performed on the healthy control group absent a diagnosis of diabetes.
   - ➢ FA using the widest approach available (e.g., Optos ultra-widefield imaging device, if available) (within prior 8 days of ocular examination).

- *The eye with the higher level of DR should always be the transit (rapid series) eye. If both eyes are of equal DR severity, the right eye should be the transit eye.*

7. Ocular examination on each eye including slit lamp, measurement of intraocular pressure, lens assessment, and dilated ophthalmoscopy *(on day of visit)*.

8. Physical examination to include:

   - Weight and height *(within prior 8 days)*
   - Blood pressure *(within prior 30 days)*

9. ***Fasting*** blood draw for:

   - HbA1c, liver function tests (LFTs), serum creatinine (used for estimated glomerular filtration rate [eGFR]), creatine kinase (CK), complete blood counts (CBC), and lipid panels (*within prior 30 days of screening visit, or up to 30 days after screening visit)*

     - *The central laboratory must be used – see AFA Coordinator Manual for collection procedure. Includes one sample for HbA1c and CBC tests and one sample for extraction of serum for remaining tests above.*
     - *If HbA1c value is not obtained from central laboratory because sample cannot be analyzed, it does not need to be repeated if available in the prior 3 months.*
     - *A small snack will be available for the participant after blood draws are performed.*

   - De-identified serum sample for metabolite measurements (e.g., deoxysphingolipids, serine, other amino acids) using liquid chromatography–mass spectrometry (LC-MS). (*within prior 30 days of screening visit, or up to 30 days after screening visit)*

     - *Includes two 10 mL tubes (or three 7 mL tubes, dependent on the centrifuge) that will be centrifuged for serum extraction and shipped on dry ice. See laboratory procedure manual for details regarding collection, processing, handling, and shipping procedure.*

   - Whole blood for genetic testing under this protocol

     Note: this is collection of blood for DNA extraction as part of AFA, the participant can be separately consented to allow this DNA sample to be included in the ongoing repository project (Protocol GEN) but only 1 DNA sample is collected. If the participant does not consent for their sample to be part of the repository (Protocol GEN), then the DNA sample will be destroyed after Protocol AFA analyses end.

10. Urine sample (*within prior 30 days of screening visit, or up to 30 days after screening visit)* for:

    a. Creatinine and albumin

    b. Pregnancy test for all biological females who are premenopausal and are not surgically sterile

11. Additional ancillary testing procedures on each eye (only obtained by a subset of sites) include:

    a. OCT angiography using Heidelberg Spectralis *(within prior 8 days of ocular exam)*

    b. Contrast sensitivity using the study-provided AST Manifold® *(within prior 8 days of ocular exam)*

3.2 Adverse Event Reporting

Only adverse events related to AFA cohort study participation that required medical intervention will be reported. Bruising or pain at the site of a venipuncture or fingerstick will not be reported as an adverse event unless severe enough that treatment is needed.

If a participant is enrolled in the parent Protocols AF and GENES, then the AE will be reported under the Protocol AF or Protocol-GENES respectively.

3.3 Miscellaneous Considerations

3.3.1 Participant Withdrawal

Participation in the study is voluntary, and a participant may withdraw without completing the study. For participants who withdraw, their data will be used up until the time of withdrawal.

3.3.2 Confidentiality

For security and confidentiality purposes, participants will be assigned an identifier that will be used instead of their name. Protected health information gathered for this study will be shared with the Coordinating Center, the Jaeb Center for Health Research in Tampa, FL. De-identified participant information may also be provided to research sites involved in the study.

The samples to characterize the participants (e.g., HbA1c, LFTs, urine, etc.) will be analyzed at the University of Minnesota, Advanced Research and Diagnostic Laboratory and will not be stored, maintained, and/or used for future research. The samples for metabolomics research will be sent to the central laboratory until a sufficient number has been collected to provide to LMRI in batches. Once provided to LMRI, the de-identified serum samples may be stored, maintained and used for future research. They may also provide de-identified samples to other researchers and industry collaborators.

The whole blood samples for genetic analyses may be sent to one or more LMRI contracted laboratory (e.g., Biospecimen Processing and Biorepository (BPB) at Fred Hutchinson Cancer Center) facilities within the United States for SNP typing and targeted gene analysis as it pertains to DR. These samples can only be used to perform analyses to meet the research objectives under this protocol. These samples are not permitted to be stored, maintained, and/or used for future research, cannot be used for any other purposes outside of this research protocol, cannot be shared with other researchers or entities outside of LMRI and the laboratories, and may not be used to re-identify participants in accordance with a written agreement with LMRI.

3.3.3 Participant Compensation

Participant compensation will be specified in the informed consent form.

3.3.4 Study Termination

Participant recruitment and enrollment of the AFA-only cohort will end upon enrollment of approximately 200 participants among the planned groups, although enrollment in some cohorts may end sooner depending on feasibility of recruitment and/or the target goal being met

3.3.5 Quality Assurance and Monitoring

Concurrent with the parent Protocol AF and Protocol-GENES, designated personnel from the Coordinating Center (JCHR) will be responsible for maintaining quality assurance (QA) and quality control (QC) systems to ensure that the clinical portion of the trial, and genetic sample processes and procedures, are conducted and data are generated, documented and reported in compliance with the protocol, Good Clinical Practice (GCP) and the applicable regulatory requirements, as well as to ensure that the rights and wellbeing of trial participants are protected and that the reported trial data are accurate, complete, and verifiable. Informed consent processes and adverse events will be prioritized for monitoring.

Chapter 4: Statistical Considerations

The approach to sample size and statistical analyses are summarized below. A detailed statistical analysis plan (SAP) will be written in collaboration with LMRI.

4.1.1 Hypothesis

Null Hypothesis (H_0): There are no significant genetic or metabolic dysregulations associated with the development and progression of diabetic retinopathy.

Alternative Hypothesis (H_1): There exist some genetic or metabolic that are associated with the development and progression of diabetic retinopathy.

4.1.2 Sample Size

Table 1. Description of Groups for Analyses from Protocols AF & AFA

Analysis Group Number	Group Description Participants with Type 2 Diabetes, and worse eye:	Target Number of Participants
AFA Group 1	Absent of DR, or microaneurysms only (Diabetes ≥15 yrs) **(DR severity level 10 to 20)**	**80**
Protocol AF* Cohort, Group 2	Mild to moderately severe DR **(DR severity level 35 to 47)**	**200**
AFA Group 3	Severe non-proliferative DR **(DR severity level 53)**	**20**
AFA Group 4	Proliferative DR **(DR severity level 61 to 81)**	**80**
AFA Group 5	**Absent of any diagnosis of diabetes and absent evidence of retinal disease as determined by color fundus photography and OCT imaging**	**20**
Total Number of Participants in the Analysis Pool (AF & AFA)		**400**

*Eligibility is determined by having sufficient serum sample volume and DR Severity Grade, among other criteria (type 2 diabetes, Genetics collection, etc.); the final sample dataset will be adjusted, as needed, for analysis, since some over-enrollment in AFA is possible.

4.1.3 Outcome Measures

The primary outcome measure will be to measure circulating metabolites and quantify genetic markers (see 4.1.3.1) in patients of different DR severity stages (Group 1-4, Table 1) and in healthy control participants.

4.1.3.1 Genetic and Metabolic markers

Genetic markers will be quantified in the form of Single Nucleotide Polymorphisms (SNPs) via SNP-Arrays technologies.

Metabolic levels will be quantified by mass spectrometry.

4.1.3.2 DR severity level

DR severity level will be determined by the Reading Center. Both eyes of a participant will be included in the analysis. The participant's worse eye will be decisive for the final classification of a patient in the above-mentioned categories.

4.1.4 Descriptive Statistics

Data description will be presented as summarized descriptive statistics (n, mean, standard deviation [SD], median, minimum, maximum and percentages). Variables include demographic data (gender, age, ethnicity), medical history (length of diabetes, medications), HbA1c, BMI, history of neuropathy, DR severity level.

4.1.5 Main Statistical Analyses

Genetic data will be cleaned by considering call rates, heterozygosity, genetically inferred sex, genotyping batches, missingness, allele frequency, and Hardy-Weinberg disequilibrium. SNP imputation will be performed using the Michigan Imputation Server. Principal component analysis will be used to account for ancestry differences across participants. Genetic relationship between participants will be estimated and accounted for in the analyses. Polygenic risk factors will be constructed by integrating results from external studies performed on T2D, DR, and metabolic levels.

Metabolomics data will be cleaned for internal batches used in different mass-spectrometry machines. Outlying samples and metabolites will be discarded as well as metabolites detected in extremely low abundance. Missing metabolic levels will be imputed using multivariate imputation methods. Principal components analysis will be used to visually inspect the data.

To evaluate the relationships between genetic and metabolic levels with disease severity, different linear regression analyses will be performed. Depending on the data at hand the tools to perform such analyses will vary but in general these will be multivariable linear regression approaches that adjust for potential imbalances of sample covariates as well as genetic ancestry. Intra-sample correlation will be taken into account by using mixed modelling approaches. The results from these associations will be used in several downstream analyses such as enrichment, burden or over-representation analysis. Post-GWAS analyses such as eQTL, TWAS and Mendelian Randomization will also be performed on these data.

Significance Level: A significance level, α, of 0.05 will be used for statistical testing of the study endpoints. False discovery rate due to multiple testing will be addressed by correcting the p-values using the Benjamini-Hochberg adaptive false discovery rate procedure. False discovery rate threshold will be set to 5% to claim significance.

Multi-omics analysis will be performed by integrating all significant markers found across the genetic and metabolic landscapes. Multivariate co-regulation networks will be constructed to understand potential biological mechanisms underpinning DR. Multivariate clustering techniques will be used to investigate whether combinations of multi-omics factors are able to better stratify across DR severity groups. Prediction models will be built to understand potential diagnostic and prognostic tools. Linear modelling techniques will be implemented to understand potential causative networks across the different 'Omics markers as well as to assess potential interactions across different markers.

Finally, external data (such as data from UK Biobank or the MacTel consortium studies) will be integrated into our study to further expand the discovery potential for this study.

Chapter 5: REFERENCES

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