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Complete List of Authors:	Burke, Kerry; Manchester University NHS Foundation Trust, Manchester Centre for Genomic Medicine, St Mary's Hospital; The University of Manchester, Division of Evolution, Infection and Genomics, School of Biological Sciences Mirza, Selman; The University of Manchester, Centre for Biostatistics, School of Health Sciences Wright, Stuart; The University of Manchester, Manchester Centre for Health Economics Greaves, Nicholas; Manchester University NHS Foundation Trust, Manchester Vascular Centre, Manchester Royal Infirmary Newman, William; Manchester University NHS Foundation Trust, Manchester Centre for Genomic Medicine, St Mary's Hospital; The University of Manchester, Division of Evolution, Infection and Genomics, School of Biological Sciences McDermott, John; Manchester University NHS Foundation Trust, Manchester Centre for Genomic Medicine, St Mary's Hospital; The University of Manchester, Division of Evolution, Infection and Genomics, School of Biological Sciences
Keywords:	Vascular surgery < SURGERY, GENETICS, VASCULAR SURGERY

A Prospective Observational Study to Assess the Impact of Pharmacogenetics on Outcomes in Vascular Surgery (PROSPER)

Kerry A Burke[1-3], Selman Mirza[4], Stuart J Wright[5], Nicholas S Greaves[3], William G Newman[1,2] John H McDermott[1,2]

1. Manchester Centre for Genomic Medicine, St Mary's Hospital, Manchester University NHS Foundation Trust, Oxford Road, Manchester, M13 9WL.

2. Division of Evolution, Infection and Genomics, School of Biological Sciences, University of Manchester, Manchester M13 9PT.

3. Manchester Vascular Centre, Manchester Royal Infirmary, Manchester University NHS Foundation Trust, Oxford Road, Manchester, M13 9WL.

4. Centre for Biostatistics, School of Health Sciences, The University of Manchester, M13 9PL.

5. Manchester Centre for Health Economics, The University of Manchester, Manchester, M13 9PL.

Corresponding author

Kerry Burke: kerry.burke@nhs.net

Manchester Centre for Genomic Medicine, St Mary's Hospital, Manchester University NHS Foundation Trust, Oxford Road, Manchester, M13 9WL

Author contributions

KB – study concept and design, protocol writing
SM – study concept and design, protocol writing
SW – study concept and design research expertise
NG – study concept and design, research expertise
WN – study concept and design, research expertise
JD – study concept and design, protocol writing
All authors contributed to the final protocol.

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SJW is supported by a Wellcome Trust Early-Career Award (226922/Z/23/Z).

Competing interests

John McDermott and William Newman are co-founders of Fava Health.
The other authors have no competing interests.

Key words

Pharmacogenetics, vascular surgery, clopidogrel, chronic limb threatening ischaemia

Word count: 1903

Abstract

Introduction

Patients with chronic limb threatening ischaemia (CLTI) are often prescribed clopidogrel in order to reduce their risk of major adverse limb and cardiovascular events. Clopidogrel is metabolised by the CYP2C19 enzyme, and genetics variations in *CYP2C19* are common. These variants can influence an individual's ability to metabolise clopidogrel to its active metabolite. This work aims to establish the relationship between patient genotype and outcomes after revascularisation in patients with CLTI who are prescribed clopidogrel. It will consider whether pharmacogenetics can be used to ensure patients are prescribed effective medications to optimise their outcomes.

Methods and analysis

This is a prospective observational cross-sectional study of patients undergoing lower limb surgical, endovascular or hybrid revascularisation for CLTI. Patients taking clopidogrel post-procedure, as well as those prescribed a non-clopidogrel based medication regimen, will be recruited prior to or shortly after revascularisation. Patients will undergo *CYP2C19* genotyping and will be followed-up using online records.

Ethics and dissemination

Manchester University Research Ethics Committee approval as obtained was part of the Implementing Pharmacogenetics to Improve Prescribing (IPTIP) trial process (IRAS 305751). The results of the study will be published in a peer-review journal and presented at international conferences.

Registration

This work is a sub-protocol for the IPTIP study which is registered as ISRCTN14050335.

Strengths and limitations of this study

- This is a prospective observational cross-sectional study of patients undergoing lower limb revascularisation for chronic limb threatening ischaemia.
- This work aims to demonstrate the impact of *CYP2C19* variants on patient outcome after revascularisation, for which the research base is currently very limited.
- A comparator group will be included of patients prescribed non-clopidogrel based medication regimens.
- A low drop-out rate is anticipated.
- As this is an observational trial, there is no patient randomisation.

Introduction

Patients with chronic limb threatening ischaemia (CLTI) have significant lower extremity arterial disease and are known to be at high risk of major adverse limb events (MALE), including loss of vessel patency, need for surgical intervention and major amputation[1]. They are also at a significantly increased risk of major cardiovascular events (MACE), including myocardial infarction, cerebrovascular events and death[1]. Clopidogrel is an anti-platelet agent which is widely used in order to reduce the risk of both MALE and MACE in patients with CLTI[2,3]. It is a thienopyridine pro-drug which is metabolised by the CYP2C19 enzyme in the liver. Genetics variations in *CYP2C19* are common and can influence an individual's ability to metabolise clopidogrel to its active metabolite[4].

Research studies in both cardiac and stroke medicine have demonstrated significantly worse outcomes in patients treated with clopidogrel who are poor metabolisers of CYP2C19, and major guidelines have advocated a role of genetic testing in these specialties[5–7]. Research into the impact of *CYP2C19* alleles in vascular surgery is much more limited, but does suggest an association between poor metabolisers of CYP2C19 and adverse outcomes in patients taking clopidogrel[8].

This protocol describes a prospective observational cross-sectional study which aims to establish the relationship between patient genotype and outcomes after revascularisation in patients with CLTI who are prescribed clopidogrel. It will consider whether pharmacogenetics can be used to ensure patients are prescribed effective medications to optimise their outcomes.

Methods and analysis

This is a prospective observational cross-sectional study involving inpatients and outpatients at Manchester University NHS Foundation Trust (MFT), to assess whether genotype is associated with clinical outcome following revascularisation for patients with CLTI who are prescribed clopidogrel. A comparator group who are prescribed non-clopidogrel based medication regimens will also be recruited. This work is a sub-protocol for the Implementing Pharmacogenetics to Improve Prescribing trial (IPTIP) study[9].

Primary outcomes

1. Amputation-free survival at 1 year

2. Composite of Major Adverse Limb Events (MALE) in the index limb [amputation above the ankle, major limb re-intervention (graft revision, thrombectomy or thrombolysis), loss of graft/vessel patency] and death from any cause at 1 year

Secondary outcomes

1. Minor re-intervention (angioplasty, stent)

2. MALE events at any time [amputation above the ankle, major limb re-intervention (graft revision, thrombectomy or thrombolysis), loss of graft/vessel patency]

3. Total re-interventions

4. Death within 30 days

5. Major adverse cardiovascular events (MACE) events at any time (myocardial infarction, cerebrovascular event or all-cause death)

6. Rate of systemic or gastro-intestinal bleed

Inclusion criteria

- Patients awaiting revascularisation (either surgical, endovascular or hybrid) for CLTI or acute-on-chronic limb ischaemia, OR who have had a revascularisation procedure in the past 3 months
- No previous revascularisation in the study leg 3 months prior to this intervention (excluding diagnostic angiograms)
- Patients over the age of 18 years who are able to consent for themselves

Exclusion criteria

- Patients receiving long term, full-dose anticoagulation post-procedure (does not include low-dose rivaroxaban)
- Acute limb ischaemia, aneurysmal disease, vasculitis, Buerger's disease
- Patients being managed conservatively without revascularisation

- Patients who are pregnant, breastfeeding, on chemotherapy or radiotherapy
- Patients with less than six months life expectancy

Eligible participants will be provided with a patient information leaflet and will sign a consent form if they wish to enrol. A trained member of the research team will then take either a single blood sample or sputum sample, at the preference of the patient. Baseline demographic details will be collected during this time. Beyond this, participants will not be asked to do anything further for the study or to attend any future study visits. The blood or sputum sample will then be transported to the Manchester Centre for Genomic Medicine via existing and secure clinical pathways for internal specimen transport. DNA will be extracted and quantified by the North West Genomic Laboratory Hub (NW-GLH), a National Health Service (NHS) ISO15189 accredited laboratory. DNA samples will be labelled with the Study ID and stored at -20°C until genotyping. Genotyping will be undertaken using the AgenaTM iPLEX PGx 74 assay which can determine *CYP2C19* genotype status. All participants will have details about their past medical history, surgical history and prescribed medications collected from the Greater Manchester Care Record (GMCR). This information and the genetic data will then be linked in a secure database. This data will be pseudo-anonymised, meaning that the researcher analysing the data will not know whose data they are looking at.

The past medical history, surgical history and prescribed medication of participants will be recorded throughout the study period using the GMCR, NHS, MFT and other online records. The total follow-up period will be two years. Once the data collection period is complete, participant genotype will then be added to the final dataset within the secure research environment, linked by the Study ID. The final pseudonymised dataset will include the Study ID, patient demographics, past medical and surgical history, prescription record, patient genotype and metabolizer status.

Participants can withdraw consent at any time without giving any reason without their care or legal rights being affected, as participation in the research is voluntary. Patients who withdraw their consent to be part of the study will have their data removed from the final analysis pipeline and their DNA sample will be destroyed.

Sample size

The desired sample size has been calculated assuming an average primary outcome rate of 0.35 at 1 year, a hazard ratio for *CYP2C19* loss of function (LoF) allele carriers for the primary endpoint of 2.0 and prevalence of LoF allele carriers of 26.2%[10,11]. Based on a survival analysis power calculation, using a two-tailed α of 0.05 and a β of 0.1, 114 events would be needed to identify an effect. Taking into consideration the baseline event rate, the study would need to recruit 326 participants receiving a clopidogrel-based medication regimen. Assuming 75% of all admissions receive a clopidogrel based antiplatelet therapy with 10% missing data, 483 participants would need to be recruited to the study in total.

Statistical analysis

The whole cohort will be split into two groups for analysis. Group 1 involves patients prescribed a clopidogrel based regimen (such as clopidogrel monotherapy or a clopidogrel containing duel antiplatelet therapy), and Group 2 which involves patients taking a non-clopidogrel based regimen (such as aspirin or aspirin + low-dose rivaroxaban). Each group will be further subdivided into *CYP2C19* LoF allele carriers and *CYP2C19* LoF allele non-carriers (Table 1).

	CYP2C19 LoF allele carrier (26.2%)	*CYP2C19* LoF allele non-carrier (73.8%)
1. Clopidogrel containing anti-platelet therapy (75%)	**A**	**B**
2. Non-clopidogrel containing anti-platelet therapy (25%)	**C**	**D**

Table 1: Analysis Groups within the PROSPER Study. Estimated frequencies (%) in each group based on historical data are provided.

The data will be explored using descriptive statistics as follows:

1. Comparison of baseline characteristics, treatment group and median follow-up time by carrier status for all participants.
2. Flow chart of recruitment to include numbers who withdraw consent and/or missing data by carrier status and treatment group.

The hazard ratio (HR) or incidence rate ratio (IRR) with associated 95% CIs of primary and secondary outcomes will be estimated to compare carrier status (i.e. those who carry and do not carry *CYP2C19* LoF alleles). Statistical models for primary outcomes will be adjusted for *CYP2C19* gene variant carrier status, treatment group (i.e. clopidogrel or non-clopidogrel), age, gender, ethnicity, diabetes, renal disease, smoking status, limb Rutherford classification and prior intervention to the index leg. Subjects will be followed until the primary endpoint or death for any cause with censoring at 12 months. Potential competing events are defined as death from any cause and a sensitivity analysis will be included to assess competing risks for amputation-free survival at 1-year. A significance level of 5% will be used throughout.

Primary and secondary outcomes will be explored as follows:

1. The number of primary and secondary events by carrier status with crude incidence rates of events (with 95% CIs) at 1-year.
2. Kaplan-Meier (KM) plots for both primary outcomes by carrier status.
3. Cumulative Incidence Functions (CIF) and 1-KM function plots for amputation-free survival by carrier status will be compared to investigate potential competing risks[12].

Primary outcomes analysis:

1. Amputation-free survival at 1-year will be investigated using Cox regression to compare carrier status. The assumption of proportional hazards will be assessed examining Schoenfeld residuals and by including an interaction between time and carrier status in the model[13]. Proportional Hazards (PH) will also be assessed using Mantel-Haenszel methods to compare hazard ratios with the Cox model. If the PH assumption cannot be satisfied, amputation-free survival at 1-year will be analysed using Restricted Mean Survival Time(RMST)[14].

If the comparison for CIF and 1-KM functions suggests the potential for competing risks, then the analysis will examine the cause-specific hazard model, presenting results for both cause-specific HRs and sub-distribution HRs[12]. Both Cox regression and RMST can produce estimates of the HR in the presence of competing risks.

2. Composite of MALE in the index limb [amputation above the ankle, major limb re-intervention (graft revision, thrombectomy or thrombolysis)] and death from any cause at 1-year will be investigated using the same approach as amputation-free survival (excluding the competing risks analysis).

Secondary outcomes analysis:

1. MACE events at any time (MI, CVA or all-cause death) at 1-year will be analysed in the same manner as amputation-free survival (excluding the competing risks analysis).
2. Death from any cause within 30 days. Numbers will be presented by carrier status and treatment group. No formal comparison between carrier status will be made.
3. Minor re-intervention (angioplasty, stent). A zero-inflated negative binomial model will be used to examine the number of minor re-interventions at 1-year. If the alpha parameter and likelihood ratio test from this model demonstrate there is no issue with overdispersion, then minor re-interventions at 1-year will be examined using a zero-inflated Poisson model.
4. Total re-interventions will use the same approach as minor re-interventions at 1-year.
5. Rate of systemic or gastrointestinal bleed will use the same approach as minor re-interventions at 1-year.

Additional Analyses

1. Exploratory analysis for the interaction of carrier status and treatment group for both primary outcomes. The model chosen will be based on the primary analysis depending on the PH assumption and if competing risks are present for amputation-free survival at 1-year (i.e. either Cox or RMST).
2. If the level of missing data is much higher than expected, multiple imputation using chained regression equations will be used as a sensitivity analysis to compare HRs with the primary analysis.

Data statement

An anonymised version of the genetic dataset will be stored in a data repository with no limitations on access or use. Genotype data will be deposited in the Figshare repository (https://figshare.com/) at the end of the study following publication. Data will be open access and will be entirely anonymised, with no study ID.

Patient and public involvement

During protocol development, the study design and methodology were discussed with the patient and public involvement and engagement with research team, called Vocal, at MFT. This was done in a focus group session. The results of the study will be provided back to the Vocal group. Patients participating in the study are provided with an option on the consent form to receive a summary of the study results.

Ethics and dissemination

Manchester University Research Ethics Committee approval as obtained was part of the IPTIP trial process (IRAS 305751). The results of the study will be published in a peer-review journal and presented at international conferences.

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Corresponding author

Kerry Burke: kerry.burke@nhs.net

Manchester Centre for Genomic Medicine, St Mary's Hospital, Manchester University NHS Foundation Trust, Oxford Road, Manchester, M13 9WL

Author contributions

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SW – study concept and design research expertise
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All authors contributed to the final protocol.

Guarantor is William Newman.

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Abstract

Introduction

Patients with chronic limb threatening ischaemia (CLTI) are often prescribed clopidogrel in order to reduce their risk of major adverse limb and cardiovascular events. Clopidogrel is metabolised by the CYP2C19 enzyme, and genetics variations in *CYP2C19* are common. These variants can influence an individual's ability to metabolise clopidogrel to its active metabolite. Few studies have investigated the relationship between patient genotype and outcomes in vascular surgery. This work aims to establish the relationship between patient genotype and outcomes after revascularisation in patients with CLTI who are prescribed clopidogrel. It will consider whether pharmacogenetics can be used to ensure patients are prescribed effective medications to optimise their outcomes.

Methods and analysis

This is a prospective observational cohort study of patients undergoing lower limb surgical, endovascular or hybrid revascularisation for CLTI. Patients taking clopidogrel post-procedure, as well as those prescribed a non-clopidogrel based medication regimen, will be recruited prior to or shortly after revascularisation. Patients will undergo *CYP2C19* genotyping and will be followed-up using online records.

Ethics and dissemination

Manchester University Research Ethics Committee approval as obtained was part of the Implementing Pharmacogenetics to Improve Prescribing (IPTIP) trial process (IRAS 305751). The results of the study will be published in a peer-review journal and presented at international conferences.

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Strengths and limitations of this study

- This is a prospective observational cohort study of patients undergoing lower limb revascularisation for chronic limb threatening ischaemia.
- This work aims to demonstrate the impact of *CYP2C19* variants on patient outcome after revascularisation, for which the research base is currently very limited.
- A comparator group will be included of patients prescribed non-clopidogrel based medication regimens.
- A low drop-out rate is anticipated.
- As this is an observational trial, there is no patient randomisation.

Introduction

Patients with chronic limb threatening ischaemia (CLTI) have significant lower extremity arterial disease and are known to be at high risk of major adverse limb events (MALE), including loss of vessel patency, need for surgical intervention and major amputation[1]. They are also at a significantly increased risk of major cardiovascular events (MACE), including myocardial infarction, cerebrovascular events and death[1]. Clopidogrel is an anti-platelet agent which is widely used in order to reduce the risk of both MALE and MACE in patients with CLTI[2,3]. It is a thienopyridine pro-drug which is metabolised by the CYP2C19 enzyme in the liver. Genetics variations in *CYP2C19* are common and can influence an individual's ability to metabolise clopidogrel to its active metabolite[4].

Research studies in both cardiac and stroke medicine have demonstrated significantly worse outcomes in patients treated with clopidogrel who are poor metabolisers of CYP2C19, and major guidelines have advocated a role of genetic testing in these specialties[5–7]. Research into the impact of *CYP2C19* alleles in vascular surgery is much more limited, but does suggest an association between poor metabolisers of CYP2C19 and adverse outcomes in patients taking clopidogrel[8,9].

This protocol describes a prospective observational cohort study which aims to establish the relationship between patient genotype and outcomes after revascularisation in patients with CLTI who are prescribed clopidogrel. It will consider whether pharmacogenetics can be used to ensure patients are prescribed effective medications to optimise their outcomes.

Methods and analysis

This is a prospective observational cohort study involving inpatients and outpatients at Manchester University NHS Foundation Trust (MFT), to assess whether genotype is associated with clinical outcome following revascularisation for patients with CLTI who are prescribed clopidogrel. CLTI is defined as lower extremity ischaemic rest pain and/or tissue loss present for more than two weeks[10]. A comparator group who are prescribed non-clopidogrel based medication regimens will also be recruited. This work is a sub-protocol for the Implementing Pharmacogenetics to Improve Prescribing trial (IPTIP) study[11].

Primary outcomes

1. Amputation-free survival at 1 year

2. Composite of Major Adverse Limb Events (MALE) in the index limb [amputation above the ankle, major limb re-intervention (graft revision, thrombectomy or thrombolysis), loss of graft/vessel patency] and death from any cause at 1 year

Secondary outcomes

1. Minor re-intervention (angioplasty, stent)

2. MALE events [amputation above the ankle, major limb re-intervention (graft revision, thrombectomy or thrombolysis), loss of graft/vessel patency] at one year

3. Total re-interventions

4. Death within 30 days

5. Major adverse cardiovascular events (MACE) events (myocardial infarction, cerebrovascular event or all-cause death) at one year

6. Rate of systemic or gastro-intestinal bleed

Inclusion criteria

- Patients awaiting revascularisation (either surgical, endovascular or hybrid) for CLTI or acute-on-chronic limb ischaemia, OR who have had a revascularisation procedure in the past 3 months
- No previous revascularisation in the study leg 3 months prior to this intervention (excluding diagnostic angiograms)
- Patients over the age of 18 years who are able to consent for themselves

Exclusion criteria

- Patients receiving long term, full-dose anticoagulation post-procedure (does not include low-dose rivaroxaban)

- Acute limb ischaemia, aneurysmal disease, vasculitis, Buerger's disease
- Patients being managed conservatively without revascularisation
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- Patients with less than six months life expectancy

Patient recruitment is expected to run from August 2023 until August 2026. Eligible participants will be provided with a patient information leaflet and will sign a consent form if they wish to enrol. A trained member of the research team will then take either a single blood sample or sputum sample, at the preference of the patient. Baseline demographic details will be collected during this time. Beyond this, participants will not be asked to do anything further for the study or to attend any future study visits. The blood or sputum sample will then be transported to the Manchester Centre for Genomic Medicine via existing and secure clinical pathways for internal specimen transport. DNA will be extracted and quantified by the North West Genomic Laboratory Hub (NW-GLH), a National Health Service (NHS) ISO15189 accredited laboratory. DNA samples will be labelled with the Study ID and stored at -20°C until genotyping. Genotyping will be undertaken using the AgenaTM iPLEX PGx 74 assay which can determine *CYP2C19* genotype status. All participants will have details about their past medical history, surgical history and prescribed medications collected from the Greater Manchester Care Record (GMCR). This information and the genetic data will then be linked in a secure database. The data will be anonymised so that researchers will not be able to identify individual patients.

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withdraw their consent to be part of the study will have their data removed from the final analysis pipeline and their DNA sample will be destroyed.

Sample size

The desired sample size has been calculated assuming an average primary outcome rate of 0.35 at 1 year[12,13], a hazard ratio for *CYP2C19* loss of function (LoF) allele carriers for the primary endpoint of 2.0 and prevalence of LoF allele carriers of 26.2%[14,15]. Based on a survival analysis power calculation, using a two-tailed α of 0.05 and a β of 0.1, 114 events would be needed to identify an effect. Taking into consideration the baseline event rate, the study would need to recruit 326 participants receiving a clopidogrel-based medication regimen. Assuming 75% of all admissions receive a clopidogrel based antiplatelet therapy with 10% missing data, 483 participants would need to be recruited to the study in total.

Statistical analysis

The whole cohort will be split into two groups for analysis. Group 1 involves patients prescribed a clopidogrel based regimen (such as clopidogrel monotherapy or a clopidogrel containing duel antiplatelet therapy), and Group 2 which involves patients taking a non-clopidogrel based regimen (such as aspirin or aspirin + low-dose rivaroxaban). Each group will be further subdivided into *CYP2C19* LoF allele carriers and *CYP2C19* LoF allele non-carriers (Table 1).

	CYP2C19 LoF allele carrier (26.2%)	*CYP2C19* LoF allele non-carrier (73.8%)
1. Clopidogrel containing anti-platelet therapy (75%)	**A**	**B**
2. Non-clopidogrel containing anti-platelet therapy (25%)	**C**	**D**

Table 1: Analysis Groups within the PROSPER Study. Estimated frequencies (%) in each group based on historical data are provided.

The data will be explored using descriptive statistics as follows:

1. Comparison of baseline characteristics, treatment group, survival time and follow-up time by carrier status for all participants. Numerical measures will be summarised by the mean, standard deviation (continuous and symmetrical), or the median and interquartile range (ordinal or not symmetrical). Categorical variables will be summarised by frequencies and percentages.
2. A flow diagram showing the number of participants screened, eligible, declined to participate, and withdrawn will be used to summarise the recruitment of participants.

The hazard ratio (HR) or incidence rate ratio (IRR) with associated 95% CIs of primary and secondary outcomes will be estimated to compare carrier status (i.e. those who carry and do not carry *CYP2C19* LoF alleles). Statistical models for primary outcomes will compare *CYP2C19* gene variant carrier status and adjust fortreatment group (i.e. clopidogrel or non-clopidogrel). Statistical models will also consider the following potential confounders: age, gender, ethnicity, diabetes, renal disease, smoking status, limb Rutherford classification and prior intervention to the index leg. Subjects will be followed until the primary endpoint or death from any cause with censoring at 12 months. Potential competing events are defined as death from any cause and a sensitivity analysis will be included to assess competing risks for amputation-free survival at 1-year. A significance level of 5% and 95% confidence intervals will be used throughout.

Primary and secondary outcomes will be explored as follows:

1. The number of primary and secondary events by carrier status with crude incidence rates of events (with 95% CIs) at 1-year.
2. Kaplan-Meier (KM) plots for both primary outcomes by carrier status.
3. Cumulative Incidence Functions (CIF) and 1-KM function plots for amputation-free survival by carrier status will be compared to investigate potential competing risks[16].

Primary outcomes analysis:

1. Amputation-free survival at 1-year will be investigated using Cox regression to compare carrier status. The assumption of proportional hazards will be assessed examining Schoenfeld residuals and by including an interaction between time and carrier status in the

model[17]. Proportional Hazards (PH) will also be assessed using Mantel-Haenszel methods to compare hazard ratios with the Cox model. If the PH assumption cannot be satisfied, amputation-free survival at 1-year will be analysed using Restricted Mean Survival Time(RMST)[18].

If the comparison for CIF and 1-KM functions suggests the potential for competing risks, then the analysis will examine the cause-specific hazard model, presenting results for both cause-specific HRs and sub-distribution HRs[16]. Both Cox regression and RMST can produce estimates of the HR in the presence of competing risks.

2. Composite of MALE in the index limb [amputation above the ankle, major limb re-intervention (graft revision, thrombectomy or thrombolysis)] and death from any cause at 1-year will be investigated using the same approach as amputation-free survival (excluding the competing risks analysis).

Secondary outcomes analysis:

1. MACE events (MI, CVA or all-cause death) at 1-year will be analysed in the same manner as amputation-free survival (excluding the competing risks analysis).
2. Death from any cause within 30 days. Numbers will be presented by carrier status and treatment group. No formal comparison between carrier status will be made.
3. Minor re-intervention (angioplasty, stent). A zero-inflated negative binomial model to compare the incidence rate ratio (IRR) between carrier status will be used to examine the number of minor re-interventions at 1-year. If the alpha parameter and likelihood ratio test from this model demonstrate there is no issue with overdispersion, then minor re-interventions at 1-year will be examined using a zero-inflated Poisson model.
4. Total re-interventions will use the same approach as minor re-interventions at 1-year.
5. Rate of systemic or gastrointestinal bleed will use the same approach as minor re-interventions at 1-year.

Additional Analyses

1. Exploratory analysis for the interaction of carrier status and treatment group for both primary outcomes. The model chosen will be based on the primary analysis depending

on the PH assumption and if competing risks are present for amputation-free survival at 1-year (i.e. either Cox or RMST).

2. The frequency and percentage of missing primary outcome data will be examined by carrier status. If the level of missing data is much higher than expected, the nature of the missing data mechanism will be investigated to determine if multiple imputation with chained regression equations should be used as a sensitivity analysis to compare HRs with the primary analysis.

Data statement

An anonymised version of the genetic dataset will be stored in a data repository with no limitations on access or use. Genotype data will be deposited in the Figshare repository[19]at the end of the study following publication. Data will be open access and will be entirely anonymised, with no study ID.

Patient and public involvement

During protocol development, the study design and methodology were discussed with the patient and public involvement and engagement with research team, called Vocal, at MFT. This was done in a focus group session. The results of the study will be provided back to the Vocal group. Patients participating in the study are provided with an option on the consent form to receive a summary of the study results.

Ethics and dissemination

Manchester University Research Ethics Committee approval as obtained was part of the IPTIP trial process (IRAS 305751). The results of the study will be published in a peer-review journal and presented at international conferences.

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Protocol for an Observational Study to Assess the Impact of Pharmacogenetics on Outcomes in Vascular Surgery (PROSPER)

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Complete List of Authors:	Burke, Kerry; Manchester University NHS Foundation Trust, Manchester Centre for Genomic Medicine, St Mary's Hospital; The University of Manchester, Division of Evolution, Infection and Genomics, School of Biological Sciences Mirza, Selman; The University of Manchester, Centre for Biostatistics, School of Health Sciences Wright, Stuart; The University of Manchester, Manchester Centre for Health Economics Greaves, Nicholas; Manchester University NHS Foundation Trust, Manchester Vascular Centre, Manchester Royal Infirmary Newman, William; Manchester University NHS Foundation Trust, Manchester Centre for Genomic Medicine, St Mary's Hospital; The University of Manchester, Division of Evolution, Infection and Genomics, School of Biological Sciences McDermott, John; Manchester University NHS Foundation Trust, Manchester Centre for Genomic Medicine, St Mary's Hospital; The University of Manchester, Division of Evolution, Infection and Genomics, School of Biological Sciences
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Protocol for an Observational Study to Assess the Impact of Pharmacogenetics on Outcomes in Vascular Surgery (PROSPER)

Kerry A Burke[1-3], Selman Mirza[4], Stuart J Wright[5], Nicholas S Greaves[3], William G Newman[1,2] John H McDermott[1,2]

1. Manchester Centre for Genomic Medicine, St Mary's Hospital, Manchester University NHS Foundation Trust, Oxford Road, Manchester, M13 9WL.

2. Division of Evolution, Infection and Genomics, School of Biological Sciences, University of Manchester, Manchester M13 9PT.

3. Manchester Vascular Centre, Manchester Royal Infirmary, Manchester University NHS Foundation Trust, Oxford Road, Manchester, M13 9WL.

4. Centre for Biostatistics, School of Health Sciences, The University of Manchester, M13 9PL.

5. Manchester Centre for Health Economics, The University of Manchester, Manchester, M13 9PL.

Corresponding author

Kerry Burke: kerry.burke@nhs.net

Manchester Centre for Genomic Medicine, St Mary's Hospital, Manchester University NHS Foundation Trust, Oxford Road, Manchester, M13 9WL

Author contributions

KB – study concept and design, protocol writing
SM – study concept and design, protocol writing
SW – study concept and design research expertise
NG – study concept and design, research expertise
WN – study concept and design, research expertise
JM – study concept and design, protocol writing
All authors contributed to the final protocol.

Guarantor is William Newman.

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Competing interests

John McDermott and William Newman are co-founders of Fava Health.
The other authors have no competing interests.

Key words

Pharmacogenetics, vascular surgery, clopidogrel, chronic limb threatening ischaemia

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Abstract

Introduction

Patients with chronic limb threatening ischaemia (CLTI) are often prescribed clopidogrel in order to reduce their risk of major adverse limb and cardiovascular events. Clopidogrel is metabolised by the CYP2C19 enzyme, and genetics variations in *CYP2C19* are common. These variants can influence an individual's ability to metabolise clopidogrel to its active metabolite. Few studies have investigated the relationship between patient genotype and outcomes in vascular surgery. This work aims to establish the relationship between patient genotype and outcomes after revascularisation in patients with CLTI who are prescribed clopidogrel. It will consider whether pharmacogenetics can be used to ensure patients are prescribed effective medications to optimise their outcomes.

Methods and analysis

This is an observational cohort study of patients undergoing lower limb surgical, endovascular or hybrid revascularisation for CLTI at Manchester University NHS Foundation Trust. Patients taking clopidogrel post-procedure, as well as those prescribed a non-clopidogrel based medication regimen, will be recruited prior to or shortly after revascularisation. Patients will undergo *CYP2C19* genotyping and will be followed-up using online records. The study has 90% power to detect 114 amputations with a target sample size of 483 participants. The primary outcomes are risk of amputation at one year and a composite endpoint for the risk of major adverse limb events (MALE) or death from any cause at one year. Secondary outcomes are risk of MALE at one year, risk of major adverse cardiovascular events (MACE) or death from any cause at one year, death within 30 days of revascularisation, minor re-interventions at one year, total number of re-interventions at one year and rate of systemic or gastrointestinal bleed at one year.

Risk of amputation, MALE and MACE will be analysed using Cox models. All remaining outcomes will be analysed using negative binomial models. Potential competing events for risk of amputation will be investigated as part of a sensitivity analysis. Patients given a non-clopidogrel based medication will be compared as an additional analysis.

Ethics and dissemination

Manchester University Research Ethics Committee approval as obtained was part of the Implementing Pharmacogenetics to Improve Prescribing (IPTIP) trial process (IRAS 305751). The results of the study will be published in a peer-review journal and presented at international conferences.

Registration

This work is a sub-protocol for the IPTIP study which is registered as ISRCTN14050335.

Strengths and limitations of this study

- A low attrition rate is anticipated as patient information will be extracted from e-health records, which will increase the validity of results.
- A comparator group who are prescribed a non-clopidogrel based medication will allow us to investigate whether these patients outcomes differed from those prescribed a clopidogrel based medication during the same time period.
- As this is an observational study, there is no patient randomisation and estimates of risk are likely to be confounded by important patient characteristics.

Introduction

Patients with chronic limb threatening ischaemia (CLTI) have significant lower extremity arterial disease and are known to be at high risk of major adverse limb events (MALE), including loss of vessel patency, need for surgical intervention and major amputation[1]. They are also at a significantly increased risk of major cardiovascular events (MACE), including myocardial infarction, cerebrovascular events and death[1]. Clopidogrel is an anti-platelet agent which is widely used in order to reduce the risk of both MALE and MACE in patients with CLTI[2,3]. It is a thienopyridine pro-drug which is metabolised by the CYP2C19 enzyme in the liver. Genetics variations in *CYP2C19* are common and can influence an individual's ability to metabolise clopidogrel to its active metabolite[4].

Research studies in both cardiac and stroke medicine have demonstrated significantly worse outcomes in patients treated with clopidogrel who are poor metabolisers of CYP2C19, and major guidelines have advocated a role of genetic testing in these specialties[5–7]. Research into the impact of *CYP2C19* alleles in vascular surgery is much more limited, but does suggest an association between poor metabolisers of CYP2C19 and adverse outcomes in patients taking clopidogrel[8,9].

This protocol describes an observational cohort study which aims to establish the relationship between patient genotype and outcomes after revascularisation in patients with CLTI who are prescribed clopidogrel. It will consider whether pharmacogenetics can be used to ensure patients are prescribed effective medications to optimise their outcomes.

Methods and analysis

This is an observational cohort study involving inpatients and outpatients at Manchester University NHS Foundation Trust (MFT), to assess whether genotype is associated with clinical outcome following revascularisation for patients with CLTI who are prescribed clopidogrel. CLTI is defined as lower extremity ischaemic rest pain and/or tissue loss present for more than two weeks[10]. A comparator group who are prescribed non-clopidogrel based medication regimens will also be recruited. A statistical analysis plan has been provided as a supplement

to this article. This work is a sub-protocol for the Implementing Pharmacogenetics to Improve Prescribing trial (IPTIP) study[11].

Primary outcomes

1. Risk of amputation at 1 year, defined as the time to amputation from revascularisation.

2. Composite of Major Adverse Limb Events (MALE) in the index limb [amputation above the ankle, major limb re-intervention (graft revision, thrombectomy or thrombolysis), loss of graft/vessel patency] or death from any cause at 1 year

Secondary outcomes

1. Minor re-intervention (angioplasty, stent)

2. MALE events [amputation above the ankle, major limb re-intervention (graft revision, thrombectomy or thrombolysis), loss of graft/vessel patency] at one year

3. Total re-interventions

4. Death within 30 days

5. Major adverse cardiovascular events (MACE) events (myocardial infarction, cerebrovascular event or all-cause death) at one year

6. Rate of systemic or gastro-intestinal bleed

Inclusion criteria

- Patients awaiting revascularisation (either surgical, endovascular or hybrid) for CLTI or acute-on-chronic limb ischaemia, OR who have had a revascularisation procedure in the past 3 months
- No previous revascularisation in the study leg 3 months prior to this intervention (excluding diagnostic angiograms)
- Patients over the age of 18 years who are able to consent for themselves

Exclusion criteria

- Patients receiving long term, full-dose anticoagulation post-procedure (does not include low-dose rivaroxaban)
- Acute limb ischaemia, aneurysmal disease, vasculitis, Buerger's disease
- Patients being managed conservatively without revascularisation
- Patients who are pregnant, breastfeeding, on chemotherapy or radiotherapy
- Patients with less than six months life expectancy

Patient recruitment is expected to run from August 2023 until August 2026. Eligible participants will be provided with a patient information leaflet and will sign a consent form if they wish to enrol. A trained member of the research team will then take either a single blood sample or sputum sample, at the preference of the patient. Baseline demographic details will be collected during this time. Beyond this, participants will not be asked to do anything further for the study or to attend any future study visits. The blood or sputum sample will then be transported to the Manchester Centre for Genomic Medicine via existing and secure clinical pathways for internal specimen transport. DNA will be extracted and quantified by the North West Genomic Laboratory Hub (NW-GLH), a National Health Service (NHS) ISO15189 accredited laboratory. DNA samples will be labelled with the Study ID and stored at -20°C until genotyping. Genotyping will be undertaken using the AgenaTM iPLEX PGx 74 assay which can determine *CYP2C19* genotype status. All participants will have details about their past medical history, surgical history and prescribed medications collected from the Greater Manchester Care Record (GMCR). This information and the genetic data will then be linked in a secure database. The data will be anonymised so that researchers will not be able to identify individual patients.

The past medical history, surgical history and prescribed medication of participants will be recorded throughout the study period using the GMCR, NHS, MFT and other online records. The total follow-up period will be two years. Once the data collection period is complete, participant genotype will then be added to the final dataset within the secure research environment, linked by the Study ID. The final anonymised dataset will include the Study ID, patient demographics, past medical and surgical history, prescription record, patient genotype and metabolizer status.

Participants can withdraw consent at any time without giving any reason without their care or legal rights being affected, as participation in the research is voluntary. Patients who withdraw their consent to be part of the study will have their data removed from the final analysis pipeline and their DNA sample will be destroyed.

Data statement

An anonymised version of the genetic dataset will be stored in a data repository with no limitations on access or use. Genotype data will be deposited in the Figshare repository[12] at the end of the study following publication. Data will be open access and will be entirely anonymised, with no study ID.

Patient and public involvement

During protocol development, the study design and methodology were discussed with the patient and public involvement and engagement with research team, called Vocal, at MFT. This was done in a focus group session. The results of the study will be provided back to the Vocal group. Patients participating in the study are provided with an option on the consent form to receive a summary of the study results.

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Statistical Analysis Plan

Full/ Long title of the Study: *Protocol for an Observational Study to Assess the Impact of Pharmacogenetics on Outcomes in Vascular Surgery*

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Roles and responsibility:

Chief Investigator:
Name: Professor William Newman
Manchester Centre for Genomic Medicine
Manchester University NHS Foundation Trust

Co-Investigator:
Name: Dr Kerry Burke
Manchester Vascular Centre
Manchester University NHS Foundation Trust

Co-Investigator:
Name: Dr John McDermott
Manchester Centre for Genomic Medicine
Manchester University NHS Foundation Trust

Co-Investigator:
Name: Dr Nicholas Greaves
Manchester Vascular Centre
Manchester University NHS Foundation Trust

Health Economist:
Name: Dr Stuart Wright
Manchester Centre for Health Economics
University of Manchester

Statistician:
Name: Mr Selman Mirza
Centre for Biostatistics
University of Manchester

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ABBREVIATIONS

CIF	Cumulative Incidence Function
CLTI	Chronic Limb Threatening Ischaemia
CVA	CerebroVascular Accident
DNA	DeoxyriboNucleic Acid
GI	Gastro-Intestinal
IRR	Incidence Rate Ratio
KM	Kaplan-Meier
LoF	Loss-of-Function
LTFU	Lost-To-Follow-Up
MACE	Major Adverse Cardiovascular Events
MALE	Major Adverse Limb Events
MFT	Manchester University NHS Foundation Trust
MI	Myocardial Infarction
MVC	Manchester Vascular Centre
PH	Proportional Hazards
RMST	Restricted Mean Survival Time

1 INTRODUCTION

1.1 Background and rationale

Patients with chronic limb threatening ischaemia (CLTI) have significant lower extremity arterial disease and are known to be at high risk of major adverse limb events (MALE), including loss of vessel patency, need for surgical intervention and major amputation[1]. They are also at a significantly increased risk of major cardiovascular events (MACE), including myocardial infarction, cerebrovascular events and death[1]. Clopidogrel is an anti-platelet agent which is widely used in order to reduce the risk of both MALE and MACE in patients with CLTI[2,3]. It is a thienopyridine pro-drug which is metabolised by the *CYP2C19* enzyme in the liver. Genetics variations in *CYP2C19* are common and can influence an individual's ability to metabolise clopidogrel to its active metabolite[4].

Research studies in both cardiac and stroke medicine have demonstrated significantly worse outcomes in patients treated with clopidogrel who are poor metabolisers of *CYP2C19*, and major guidelines have advocated a role of genetic testing in these specialties[5–7]. Research into the impact of *CYP2C19* alleles in vascular surgery is much more limited, but does suggest an association between poor metabolisers of *CYP2C19* and adverse outcomes in patients taking clopidogrel[8].

PROSPER is an observational cohort study which aims to establish the relationship between patient genotype and outcomes after revascularisation in patients with CLTI who are prescribed clopidogrel. It will consider whether pharmacogenetics can be used to ensure patients are prescribed effective medications to optimise their outcomes.

1.2 Objectives

The primary objective is to assess whether genotype is associated with clinical outcome following vascular intervention for individuals prescribed clopidogrel.

The secondary objective is to establish a genotyped cohort of patients presenting with PAD undergoing endovascular or surgical intervention.

The additional objective is to assess whether clinical outcome following vascular intervention for individuals prescribed clopidogrel differs to individuals prescribed other medication and how this interacts with each genotyped cohort.

2 METHODS

2.1 Study design

The study will be an observational cohort study which includes patients recruited both retrospectively and prospectively. The cohort is split into four groups based on genotype and drug treatment (see table 1 appendix B).

2.2 Sample size

The desired sample size has been calculated assuming an average amputation rate of 0.35 at 1 year[11], a hazard ratio for *CYP2C19* loss of function (LoF) allele carriers for the primary endpoint of 2.0 and prevalence of LoF allele carriers of 26.2%[12,13]. Based on the Schoenfeld method for proportional hazards[14] (PH), the power calculation using a significance level of 5% and 90% statistical power, 114 events would be needed to identify an effect. Taking into consideration the average amputation rate, the study would need to recruit 326 participants receiving a clopidogrel-based medication regimen. Assuming 25% of all admissions receive a non-clopidogrel based antiplatelet therapy with 10% missing data, 483 participants would need to be recruited to the study in total. Sample size calculations were performed in R software (version 4.4.2).

2.3 Timing of outcome assessments

For time-to-event outcomes, events will be recorded when they occur throughout the study period. All remaining outcomes will be assessed at 1 year from baseline.

2.4 Timing of final analysis

Analyses will take place after the 1-year follow-up period has been completed. All outcomes will be analysed collectively at the time of final data *identification and extraction* which will be September 2027, with all information entered into the database and the full database is cleaned and locked.

3 STATISTICAL PRINCIPLES

3.1 Confidence intervals (CI) and level of statistical significance

Analyses will be conducted using two-sided 95% confidence intervals at the 5% significance level. No formal adjustments will be made for multiplicity since each primary outcome uses a different hypothesis.

3.2 Analysis populations

The analysis population for each outcome will be the complete-case population (i.e. excluding any patients for whom data are or become unavailable for research).

The data analyses will be based on anonymised patient-level data. Patients who wish to withdraw their consent to allow their data to be used will not be included in the analyses.

4 STUDY POPULATION

4.1 Screening

Eligible participants will be identified by the reviewing surgeon and will be approached by their direct clinical or research team (CRN nursing support and sub-investigators), the study will be discussed, and a patient information leaflet (PIL) will be provided. Thereafter, if the patients wish to enrol, a consent form will be signed, blood samples for DNA will be taken and the initial recruitment proforma will be completed.

4.2 Eligibility

Inclusion criteria:

1. Patients awaiting revascularisation (either surgical, endovascular or hybrid) for CLTI or acute-on-chronic limb ischaemia, OR who have had a revascularisation procedure in the past 3 months.
2. No previous revascularisation in the study leg 3 months prior to this intervention (excluding diagnostic angiograms).
3. Patients over the age of 18 years who are able to consent for themselves.

Exclusion criteria:

1. Patients receiving long term, full-dose anticoagulation post-procedure (does not include low-dose rivaroxaban).
2. Acute limb ischaemia, aneurysmal disease, vasculitis, Buerger's disease.
3. Patients being managed conservatively without revascularisation.
4. Patients who are pregnant, breastfeeding, on chemotherapy or radiotherapy.
5. Patients with less than six months life expectancy.

4.3 Recruitment

We plan to recruit participants from the Manchester Vascular Centre (MVC) at the Manchester Foundation Trust (MFT), a specialist unit which covers a population of 2.6million and performs over 600 interventions each year.

A flow diagram showing the number of participants screened, eligible, declined to participate, and withdrawn will be used to summarise the recruitment of participants.

4.4 Withdrawal/follow-up

Patients can withdraw consent for their data to be used in the analyses. In these cases, patients will be summarised and excluded from the analyses.

Patients that withdraw consent will be censored from the date of withdrawal. Patients that are lost-to-follow-up (LTFU) will be censored from the date of last known contact.

4.5 Baseline patient characteristics

We will use the participants date of revascularisation as the baseline time of entry to the study. Comparison of baseline characteristics, treatment group, time to amputation and follow-up time by carrier status for all participants. Numerical measures will be summarised by the mean, standard deviation (continuous and symmetrical), or the median and interquartile range (ordinal or not symmetrical). Categorical variables will be summarised by frequencies and percentages. Characteristics between carrier status groups will be compared using t-tests or chi-square tests as appropriate. Baseline characteristics will include:

- Age (years)
- Gender
- Ethnicity
- Diabetes mellitus
- Renal disease
- Smoking status
- Limb Rutherford classification
- Prior intervention to the index leg

5 ANALYSIS

5.1 Outcome definitions

5.1.1 Primary outcomes

a. Risk of amputation at 1-year. Time to amputation will be measured in months from the date of revascularisation. Subjects will be followed until the primary endpoint, with censoring at time of death from any cause, withdrawal, LTFU or at 1-year.
b. Composite of Major Adverse Limb Events (MALE) in the index limb [amputation above the ankle, major limb re-intervention (graft revision, thrombectomy or

thrombolysis), loss of graft/vessel patency] or death from any cause at 1-year. Subjects will be followed until the composite endpoint, with censoring at time of withdrawal, LTFU or at 1-year.

5.1.2 Secondary outcomes

a. MALE in the index limb at 1-year. Subjects will be followed until the composite endpoint, with censoring at time of death from any cause, withdrawal or LTFU at 1-year.
b. MACE events at 1-year (MI, CVA or all-cause death). Subjects will be followed until the outcome endpoint, with censoring at time of withdrawal, LTFU or at 1-year.
c. Death within 30 days from revascularisation.
d. Minor re-interventions (angioplasty, stent) at 1-year.
e. Total number of re-interventions at 1-year.
f. Rate of systemic or gastro-intestinal (GI) bleed at 1-year.

5.2 Analysis methods

All primary and secondary outcomes will be compared by carrier status for the clopidogrel group (i.e. A vs B, see table 1 appendix B). The number of primary and secondary events by carrier status will be reported with crude incidence rates of events (with 95% CIs) at 1-year. The Cumulative Incidence Function (CIF) will be used to estimate incidence rates to account for potential competing risks[15].

5.2.1 Primary outcome analysis

a. Risk of amputation at 1-year will be estimated using Cox regression to compare carrier status for the clopidogrel group.

   The primary analysis will be adjusted for age, gender, diabetes status and Rutherford classification for severity. The hazard ratio (HR) with corresponding 95% confidence intervals (CIs) and p-values will be reported.

   The assumption of proportional hazards (PH) will be assessed by examining the Schoenfeld residuals.

b. Composite of MALE in the index limb or death from any cause at 1-year will be investigated using the same approach as risk of amputations.

5.2.2 Secondary outcome analysis

a. MALE events will be analysed in the same manner as the corresponding primary outcome but death from any cause will be censored.

b. MACE events at 1-year will be analysed in the same manner as the primary outcome for risk of amputations.
c. Death within 30 days from revascularisation. Frequency with percentages will be presented by carrier status. No formal comparison between carrier status will be made.
d. Minor re-interventions at 1-year will be analysed using a zero-inflated negative binomial model to compare carrier status. The IRR with corresponding 95% CIs and p-values will be reported.
e. Total re-interventions at 1-year will use the same approach as minor re-interventions.
f. Rate of systemic or GI bleed at 1-year will use the same approach as minor re-interventions.

5.2.3 Alternative methods

If the PH assumption cannot be satisfied, the primary outcome at 1-year will be analysed using Restricted Mean Survival Time (RMST)[16]. Both Cox regression and RMST can produce estimates of the HR in the presence of competing risks.

For secondary outcomes measured as count data, if the 'alpha' parameter and likelihood ratio test from the negative binomial model suggests no issue with overdispersion, then all relevant secondary outcomes at 1-year will be examined using a zero-inflated Poisson model.

5.2.4 Sensitivity analysis

Potential competing events are defined as death from any cause and a sensitivity analysis will be included for the risk of amputation at 1-year. CIF and 1-KM function plots for the primary outcome will be compared to investigate potential competing risks. If this comparison suggests the potential for competing risks, then the sensitivity analysis will examine the sub-distribution hazard model[15]. We will report the HR with the corresponding 95% CIs and p-values.

Baseline characteristics that do not form part of the primary analysis will be included in the sensitivity analysis and estimates of risk will be compared to the primary analysis.

5.3 Missing data

The frequency and percentage of missing primary outcome data will be examined by carrier status. If the level of missing data is much higher than expected, the nature of the missing data mechanism will be investigated. We anticipate the level of missing data in our study to be low. Missing data that could be considered at least 'missing at random' would be few and have no significant impact on the analysis. We expect the majority of our missing data to be

'not missing at random' because some patients may have to discontinue treatment with clopidogrel which implies multiple imputation methods would not be suitable.

5.4 Additional analyses

There will be a supporting analysis to examine the non-clopidogrel group to compare carrier status for the risk of amputation (i.e. C vs D, see table 1 appendix B). This treatment group will be observed during the same time period and is not expected to differ in terms of carrier status. This will provide a more robust interpretation of our results from the primary analysis. The model chosen will depend on the PH assumption and if competing risks are present for the risk of amputation at 1-year.

If our analysis for the risk of amputation shows the clopidogrel group differs in terms of carrier status, an exploratory analysis will be included to compare clopidogrel and non-clopidogrel based medications. The model chosen will depend on the PH assumption and if competing risks are present for the risk of amputation at 1-year and will make two comparisons. The first comparison will examine the clopidogrel group vs. the non-clopidogrel group for those who carry LoF alleles (i.e. A vs C, see table 1 appendix B). The second comparison will examine the clopidogrel group vs the non-clopidogrel group for non-carriers (i.e. B vs D, see table 1 appendix B).

5.5 Harms

As this is an observational study, the risk to participants and researchers is not above that of normal clinical practice. All patient DNA samples will be stored securely within an NHS facility where the risk of DNA contamination or loss is very low.

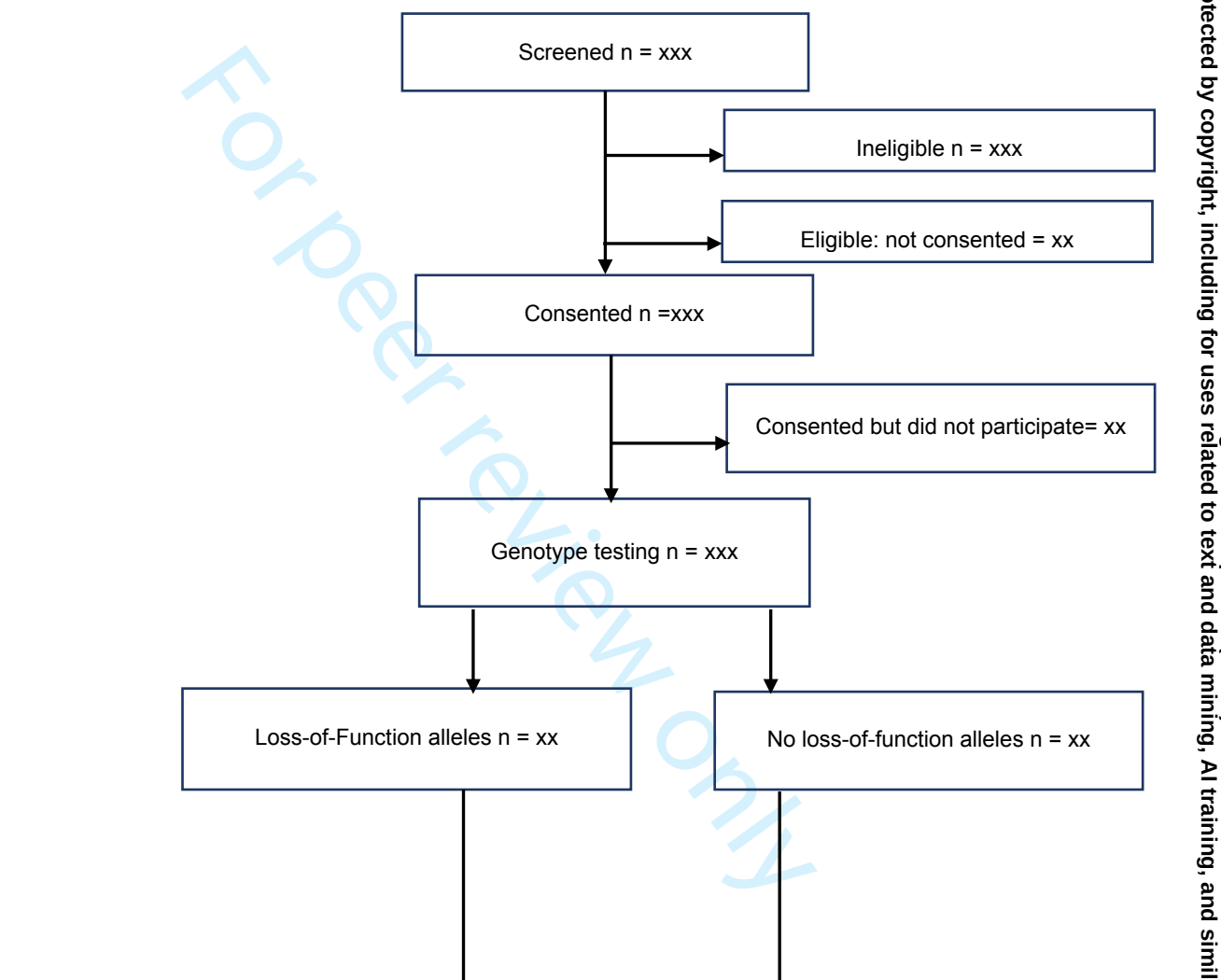
5.6 Statistical software

Statistical analyses will be performed using Stata or other similar software.

6 APPENDICES

6.1 Appendix A: Flow diagram template

Figure 1: PROSPER diagram – recruitment and follow-up of patients.



6.2 Appendix B: Analysis groups

	CYP2C19 LoF allele carrier (26.2%)	*CYP2C19* LoF allele non-carrier (73.8%)
1. Clopidogrel containing anti-platelet therapy (75%)	**A**	**B**
2. Non-clopidogrel containing anti-platelet therapy (25%)	**C**	**D**

Table 1: Analysis Groups within the PROSPER Study. Estimated percentages of carrier status in each group are based on historical data.

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