A Practical Primer in Human Complex Genetics

with a use-case in cardiovascular disease

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Contents

Chapter 1

About this primer

Ever since the first genome-wide association study (GWAS) on age-related macular degeneration, and the promise of personalized medicine in the wake of the Human Genome Project, large-scale genetic association studies hold significant sway in contemporary health research and drive drug-development pipelines. In the past 2 decades, researchers delved into GWAS, aiming to unveil genetic variations linked to both human traits, such as the color of your eyes, and rare and common complex diseases. These findings serve as crucial keys to unravel the intricate mechanisms underlying diseases, shedding light on whether the correlations identified in observational studies between risk factors and diseases are truly causal.

These studies have ushered in an exciting era where many researchers thrive on developing new methods and bioinformatic tools to parse ever-growing large datasets collected large population-based biobanks. However, the analyses of these data are challenging and it can be daunting to see the forest for tree among the many tools and their various functions. Enter *A Practical Primer in Human Complex Genetics*. This GitBook was originally written back in 2022 for the **Genetic Epidemiology** course organized by the Master Epidemiology of Utrecht University. This practical guide will teach you how to design a GWAS, perform quality control (QC), execute the actual analyses, annotate the GWAS

results, and perform further downstream post-GWAS analyses. Throughout the book you'll work with 'dummy', that is fake, data, but in the end, we will use real-world data from the first release of the *Welcome Trust Case-Control Consortium (WTCCC)* focusing on coronary artery disease (CAD).

A major component of modern-day GWAS is genetic imputation, but for practical reasons it is not part of this book. However, I will provide some pointers as to how to go about do this with minimal coding or scripting experience. Likewise, the courses does not cover the aspects of meta-analyses of GWAS, but some excellent resources exist to which I will direct. As this practical primer evolves, these and other topics may find their place in this book. I should also point out that emphasis of this book is on it being a *practical primer*. It is intended to provide some practical guidance to doing GWAS, and while theory is important, I will not cover this. Again, some very useful and excellent work exists to which I will point you, but I really want you to learn - and understand the theory - by *doing*.

So, although originally crafted as a companion for the course, this practical guide stands on its own as a comprehensive resource for diving into all facets of doing a GWAS — save for experimental follow-up, of course □ .

I can imagine this seems overwhelming, but trust me, you'll be okay. Just follow this practical. You'll learn by doing and at the end of the day, you can execute a GWAS independently.

Ready to start?

Your first point of action is to prepare your system for this course in Chapter 2.

Chapter 2

Some background reading

Standing on the shoulders of giants, that's what this book and I do. I want to acknowledge some great work that has helped me tremendously and, really, this book wouldn't exist without this awesome work. So, I do want to give you some background reading. Is it a prerequisite? No, not really. For starters, the course covers most and you'll learn as you go. And if you didn't come here through the course, you'll be fine just the same. That said, it's a always good idea to get familiar with these works as you move forward on your path towards your first GWAS - in fact, I had these printed out with markings and writings all over them as I executed my first GWAS, and they've been great as a reference

many times after.

Large parts of this work are based on four awesome Nature Protocols from the Zondervan group at the Wellcome Center Human Genetics.

1. Zondervan KT *et al. Designing candidate gene and genome-wide case-control association studies.* Nat Protoc 2007.
2. Pettersson FH *et al. Marker selection for genetic case-control association studies.* Nat Protoc 2009.
3. Anderson CA *et al. Data QC in genetic case-control association studies.* Nat Protoc 2010.
4. Clarke GM *et al. Basic statistical analysis in genetic case-control studies.* Nat Protoc 2011.

An update on the community standards of QC for GWAS can be found here:

1. Laurie CC *et al. Quality control and quality assurance in genotypic data for genome-wide association studies.* Genet Epidemiol 2010.

With respect to imputation and meta-analyses of GWAS you should also get familiar with the following two works:

1. Marchini, J. and Howie, B. *Genotype imputation for genome-wide association studies.* Nat Rev Genet 2010
2. de Bakker PIW *et al. Practical aspects of imputation-driven meta-analysis of genome-wide association studies.* Hum Mol Genet 2008.
3. Winkler TW *et al. Quality control and conduct of genome-wide association meta-analyses.* Nat Protoc 2014.

Are you ready?

Are you ready? Did you bring coffee and a good dose of energy? Let's start! Your first point of action is to prepare your system for this course in Chapter 3.

Chapter 3

Getting started

```
##
## Packages to install for this book and its contents to automagically work.
##
## * Bookdown and rmarkdown packages...
##
## * General packages...
##
## * GGplotting packages - for publication ready plotting...
##
```

* Genomic packages . . .

> [THIS CHAPTER NEEDS WORK] - introduction - briefly touch on operating system - split CoCalc vs standalone - CoCalc: everything is installed - standalone: - focus on macOS - what to install - show how to navigate on macOS Terminal [Some introductory text]

Before getting started, we need to discuss your computer. Most programs made to execute genetic epidemiology studies are developed for the Unix environment, for example Linux and macOS. So, they may not work as intended in a Windows environment. Windows does allow users to install a linux subsystem within Windows 10+ and you can find the detail guide here.

However, I highly recommend one of two options.

- One, install a linux subsystem on your Windows computer (for example a virtual machine with Ubuntu could work).
- Two, switch to macOS in combination with homebrew. This will give you all the flexibility to use Unix-based programs for your genetic epidemiology work and at the same time you'll keep the advantage of a powerful computer with a user-friendly interface.

I chose the latter.

> For this practical every command is intended for Linux/macOS, in other words Unix-systems.

CoCalc vs. Standalone For the purpose of this practical primer there are one of two steps you need to take to get started. When you are following the course, you will want to read the section **CoCalc**. When you want to use this book as a standalone, you should check out the instructions in section **Standalone** - this is probably also the section you want to follow for real-world cases.

But first, I'll briefly provide some background on the variaous programs that are commonly used.

3.1 The programs we use

We'll use a few programs throughout this practical. You'll probably need these for your (future) genetic epidemiology work too (Table 3.1).

RStudio

Table 3.1: Programs needed for genetic epidemiology.

Program	Link	Description
PLINK	https://www.cog-genomics.org/plink2/	PLINK is a free, open-source genetic analysis tool set, desig
R	https://cran.r-project.org/	A program to perform statistical analysis and visualizations.
RStudio	https://www.rstudio.com	A user-friendly R-wrap-around for code editing, debugging,
Homebrew	https://brew.sh	A great extension for Mac-users to install really useful progr

RStudio is a very user-friendly interface around R that makes your R-scripting-life a lot easier. You should get used to that. **RStudio** comes with R so you don't have to worry about that.

PLINK Right, onto PLINK.

All genetic analyses can be done in PLINK, even on your laptop, but with large datasets, for example UK Biobank size, it is better to switch to a high-performance computing cluster (HPC) like we have available at the Utrecht Science Park. The original PLINK v1.07 can be found here, but nowadays we are using a newer, faster version: **PLINK v1.9** which can be found here. It still says 'PLINK 1.90 beta' (Figure 3.1), but you can consider this version stable and save to work with, but as you can see, some functions are not supported anymore.

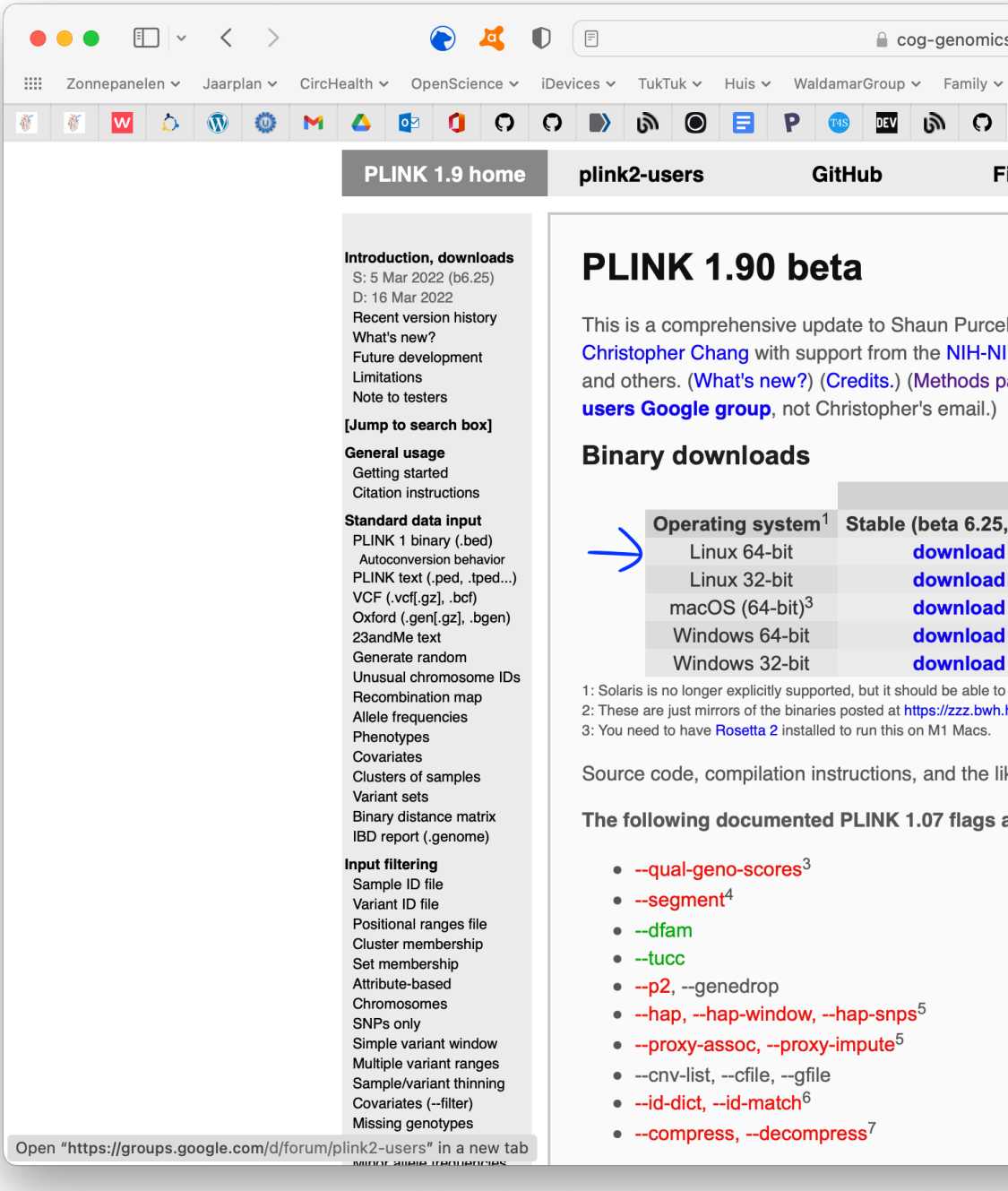


Figure 3.1: The PLINK v1.9 website.

Alternatives to PLINK

Nowadays, a lot of people also use programs like SNPTEST, BOLT-LMM, GCTA, or regenie as alternatives to execute GWAS. These programs were designed with specific use-cases in mind, for instance really large biobank data including hundreds of thousands individuals, better control for population stratification, the ability to estimate trait heritability or Fst, and so on.

Other programs

Mendelian randomization can be done either with the SMR or GSMR function from GCTA, or with R-packages, like TwoSampleMR.

3.2 CoCalc

[TEXT NEEDS UPDATING]

Now, pay attention. If you came here through the course **Genetic Epidemiology**, you don't have to do anything. All the data you need are already downloaded.

However, when you are using this book as a standalone, you'll need to start by downloading the data you need for this practical to your Desktop.

For the course we set up a CoCalc Server and everything should be fine; we installed everything you need.

3.3 Standalone

So, you plan to use this book as 'Standalone' on a macOS environment. This means you'll need to install a few things first.

3.3.1 Terminal

For all the programs we use, except **RStudio**, you will need the **Terminal**. This comes with every major operating system; on Windows it is called 'PowerShell', but let's not go there. And regardless, you will (have to start to) make your own scripts. The benefit of using scripts is that each step in your workflow is clearly stipulated and annotated, and it allows for greater reproducibility, easier troubleshooting, and scaling up to high-performance computer clusters.

Open the **Terminal**, it should be on the left in the toolbar as a little black computer-monitor-like icon. Mac users can type command + space and type terminal, a **Terminal** screen should open.

> From now on we will use little code blocks like the example to indicate a code you should type/copy-paste and hit enter. If a code is followed by a comment, it is indicated by a # - you don't need to copy-paste and execute this.

```
CODE BLOCK

CODE BLOCK # some comment here
```

3.3.2 The data you need

You'll need to start by downloading the data you need for this practical to your Desktop.

Here's the link to the data.

Link to Google Drive with data

Make sure you put the data in the ~/Desktop/practical/ folder.

The data are pretty large (approx. 15Gb), so this will take a minute or two depending on your internet connection. Time to stretch your legs or grab a coffee (data scientists don't drink tea).

3.3.3 Navigating the Terminal

You can navigate around the computer through the terminal by typing cd <path>; cd stands for "change directory" and <path> means "some_file_directory_you_want_to_go_to".

For Linux/macOS Users

will bring you to your home directory

```
cd ~
```

will bring you to the parent directory (up one level)

```
cd ../
```

will bring you to the XXX directory

```
cd XXX
```

Let's navigate to the folder you just downloaded.

```
cd ~/Desktop/practical
```

Let's check out what is inside the directory, by listing (ls) its contents.

shows files as list

```
ls -l
```

shows files as list with human readable format

```
ls -lh
```

Adding the flags –lh will get you the contents of a directory in a list (–l) and make the size 'human-readable' (–h).

shows the files as list sorted by time edited

```
ls -lt
```

shows the files as list sorted by size

```
ls -lS
```

You can also count the number of files.

```
ls | wc -l
```

And if you want to know all the function of a program simply type the following.

```
man ls
```

This will take you to a manual of the program with an extensive description of each flag (Figure 3.2).

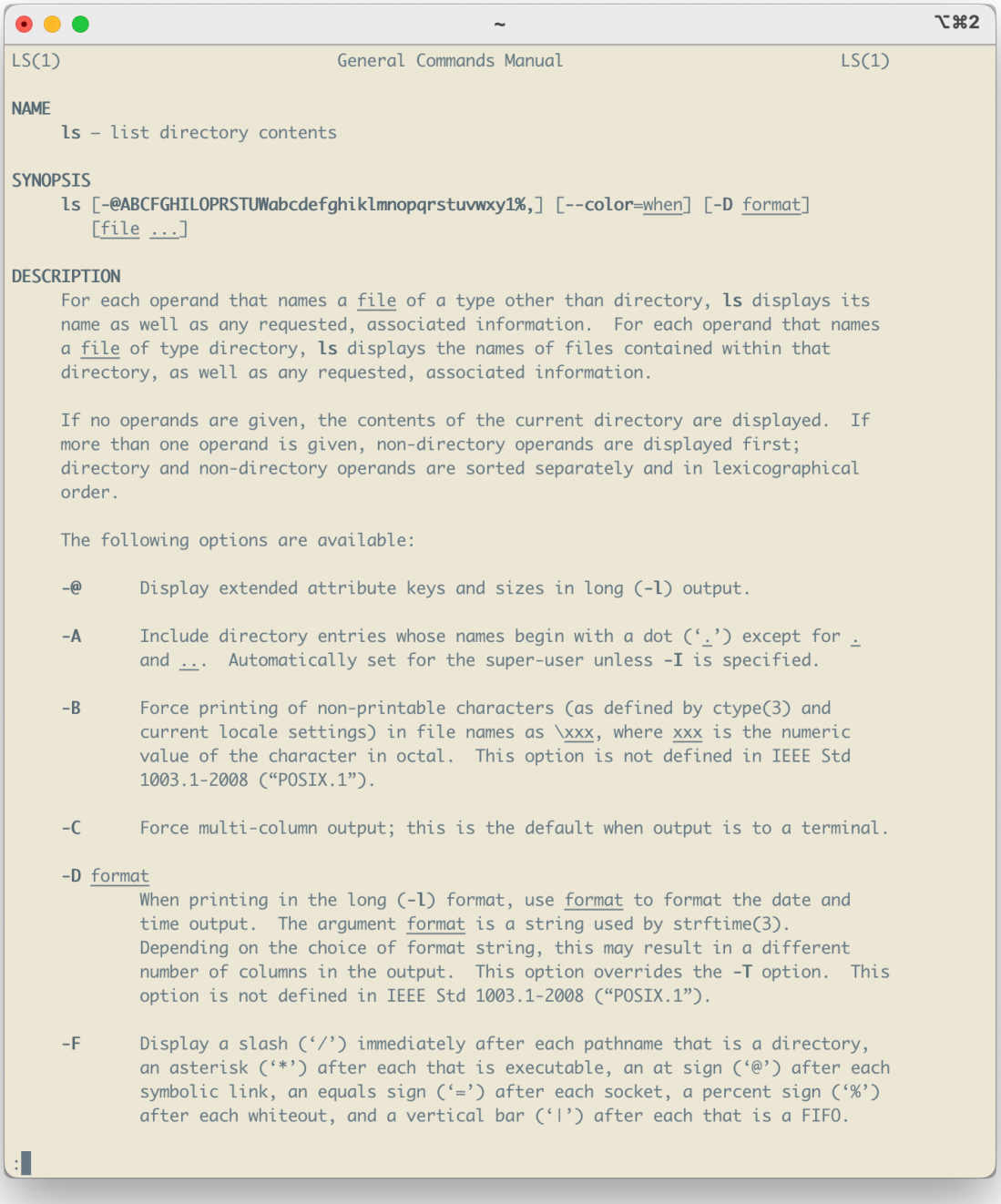


Figure 3.2: Partial output from the ls-manual.

3.3.4 Installing the software

3.3.4.1 brew

Linux has a great package-manager that is lacking on macOS. You can install brew to compensate for this. This adds the ability to install almost any Linux-based program through the **Terminal** such as wget, llvm, etc.

Open **Terminal** and execute the following:

```
/bin/bash -c "$(curl -fsSL https://raw.githubusercontent.com/Homebrew/install/HEAD/ir
```

Check if everything is in order.

```
brew doctor
```

It shouldn't report any errors.

3.3.4.2 PLINK

First, we'll get PLINK. Navigate to the **PLINK v1.9** website, which can be found here. Download the macOS (64-bit) version under 'Stable (beta x.x, day month year)'.

> Note: Apple produced Intel-based computers for a few years back, and most programs, packages, libraries and whatnot are designed for that. So, I highly recommend using software designed for that and activating Rosetta2 in your Terminal. Don't know how to do that? Following these instructions.

Unzip the folder and put plink in the practical folder.

```
mv -v ~/Downloads/plink_mac_20231211/plink ~/Desktop/practical/plink
```

3.4 Installing R and RStudio

Let's go ahead and use brew to install the R and **RStudio** software.

In **Terminal** execute the following and just follow the instructions.

```
brew install rstudio
brew install --cask r
```

Now close the terminal window - really make sure that the terminal-program has quit.

Open your fresh installation of **RStudio** by double clicking the icon. You should be seeing something like figure 3.3

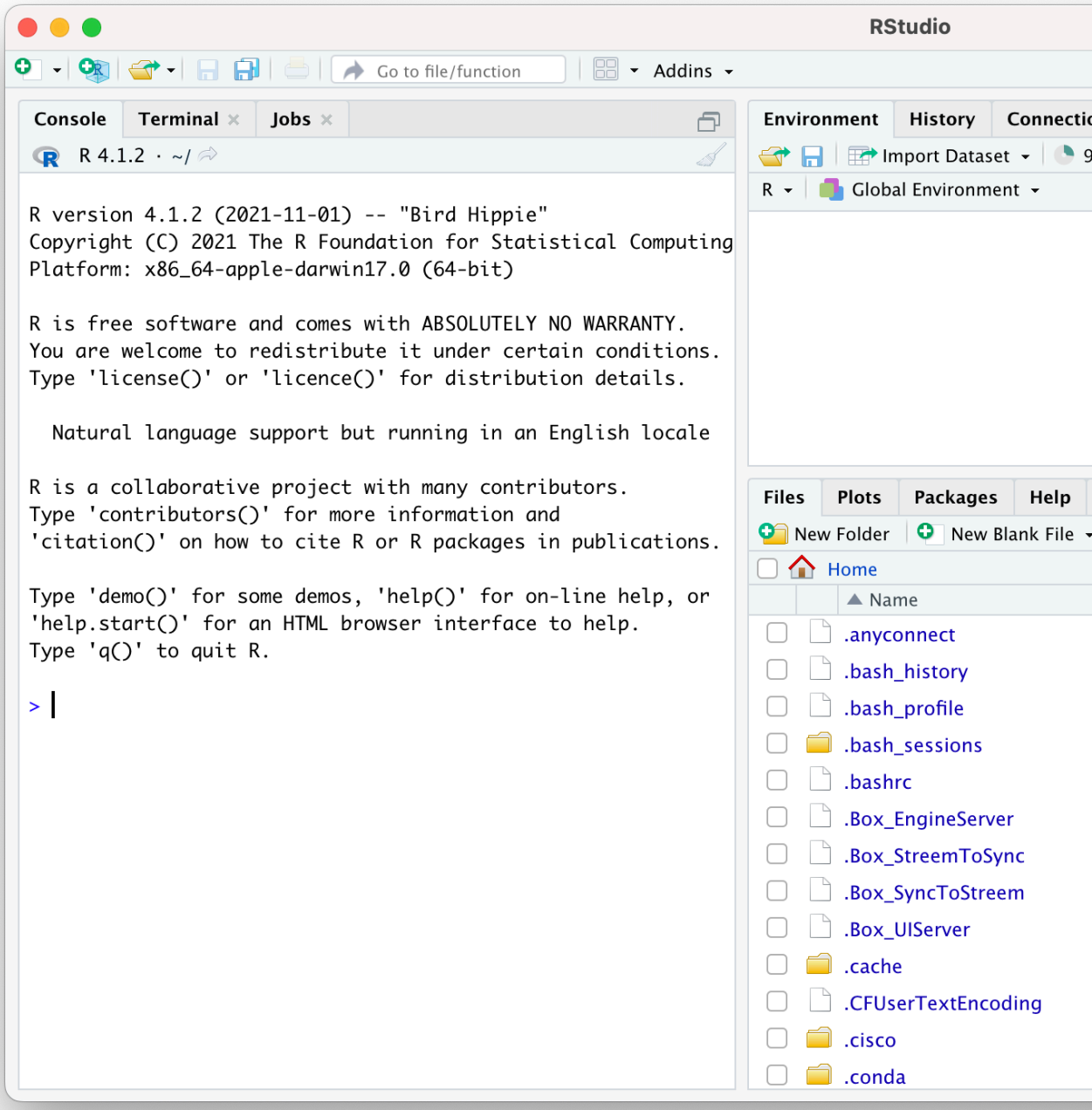


Figure 3.3: RStudio screenshot.

In the top right, you see a little green-white plus-sign, click this and select ‘R Notebook’ (Figure 3.4).

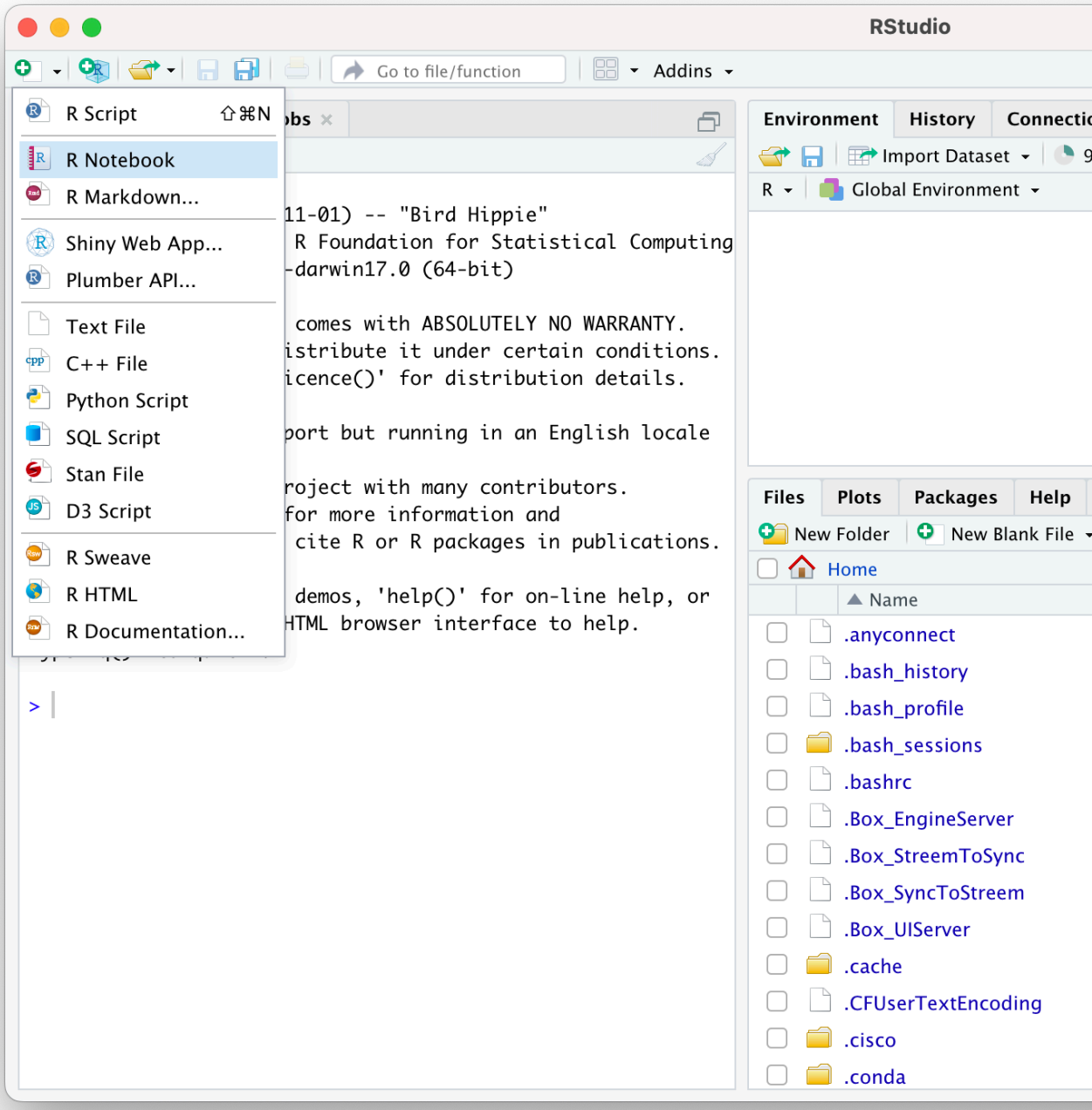


Figure 3.4: RStudio screenshot.

You will create an untitled (Untitled1) R notebook: you can combine text descriptions, like you would in a lab-journal, with code-sections. Read what is in the notebook to get a grasp on that (Figure 3.5).

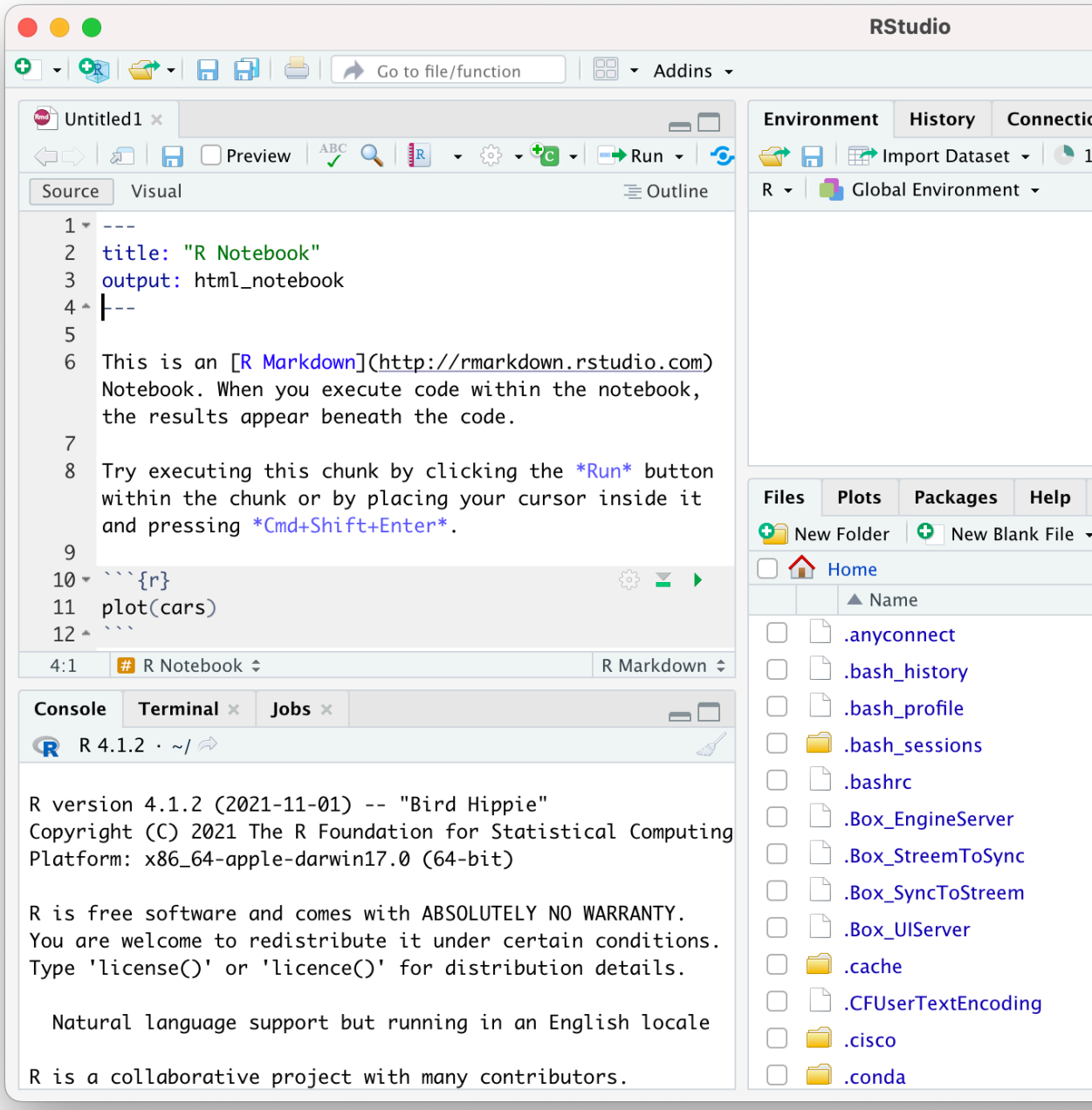


Figure 3.5: RStudio screenshot.

Right, you should be installing some packages. To do so, you can remove plot(cars) (or leave and create a new code-block as per instructions in the notebook), and copy paste the code below. Make sure to put in a code block like the example in which plot(cars) is in.

```
remotes::install_github(c("rstudio/rmarkdown"))

install.packages(c("formatR", "remotes", "httr", "usethis",
  "data.table", "devtools", "dplyr", "tibble", "tidyverse",
  "openxlsx", "ggplot2", "ggsci", "ggthemes", "qqman",
  "CMplot", "plotly", "openxlsx"))
devtools::install_github("kassambara/ggpubr")

devtools::install_github("oliviasabik/RACER")

remotes::install_github("MRCIEU/TwoSampleMR")

if (!require("BiocManager", quietly = TRUE)) install.packages("BiocManager")
BiocManager::install("geneplotter")
```

You should load these packages too.

```
library(rmarkdown)
library(formatR)

library(openxlsx)

library(data.table)

library(tibble)
library(tidyverse)
library(dplyr)
library(plotly)

library(ggplot2)
library(devtools)
library(ggpubr)
library(ggsci)
library(ggthemes)

library(qqman)
library(CMplot)
library(RACER)

library(remotes)
library(TwoSampleMR)
```

```
library("geneplotter")
```

All in all this may take some time, good moment to relax, review your notes, stretch your legs, or take a coffee.

3.5 Are you ready?

Are you ready? Did you bring coffee and a good dose of energy? Let's start!

Oh, one more thing: you can save your notebook, the one you just created, to keep all the R codes you are applying in the next chapters and add descriptions and notes. If you save this notebook you'll notice that a html-file is created. This file is a legible webbrowser-friendly version of your work and contains the codes and the output (code messages, tables, and figures). And the nice thing is, that you can easily share it with others over email.

Ok. 'Nough said, let's move on to cover some basics in Chapter 4.

Chapter 4

Steps in a Genome-Wide Association Study

Now that you understand a bit of the navigation in Unix-systems, you're ready to start this practical primer. We will make use of a dummy dataset containing cases and controls. We will explain and execute the following steps:

1. convert raw data to a more memory-efficient format
2. apply extensive quality control on samples and SNPs
3. assess the ancestral background of your study population

4. perform association testing
5. visualize association results

4.1 Converting datasets

The format in which genotype data are returned to investigators varies among genome-wide SNP platforms and genotyping centers. Usually genotypes have been called by a genotyping center and returned in the standard PED and MAP file formats designed for PLINK.

A PED file is a white space (space or tab)-delimited file in which each line represents one individual and the first six columns are mandatory and in the following order:

- 'Family ID',
- 'Individual ID',
- 'Paternal ID',
- 'Maternal ID',
- 'Sex (1=male, 2=female, 0=missing)', and
- 'Phenotype (1=unaffected, 2=affected, 0=missing)'.

The subsequent columns denote genotypes that can be any character (e.g., 1, 2, 3, 4 or A, C, G, T). Zero denotes a missing genotype. Each SNP must have two alleles (i.e., both alleles are either present or absent). The order of SNPs in the PED file is given in the MAP file, in which each line denotes a single marker and the four white-space–separated columns are chromosome (1–22, X, Y or 0 for unplaced), marker name (typically an rs number), genetic distance in Morgans (this can be fixed to 0) and base-pair position (bp units).

Let's start by using PLINK to converting the datasets to a lighter, binary form (a .bed-file). This file saves data in a more memory- and time-efficient manner (in a 'binary'-format) to facilitate the analysis of large-scale data sets (Purcell S., 2007). The marker-information is stored in the .bim-file and the family information in the .fam-file. PLINK creates a .log file (named raw–GWA–data.log) that details (among other information) the implemented commands, the number of cases and controls in the input files, any excluded data and the genotyping rate in the remaining data. This file is very useful for checking whether the software is successfully completing commands.

Make sure you are in the right directory. Do you remember how to get there?

```
cd ~/Desktop/practical
```

Next, we'll make a project directory.

```
mkdir -v ~/Desktop/practical/dummy_project
```

Now, we'll convert the .ped/.map files to the binary-format.

```
plink --file rawdata/raw-GWA-data --make-bed --out dummy_project/rawdata
```

Let's review the .log-file for a bit. It should look something like this:

```
PLINK v1.90b7.2 64-bit (11 Dec 2023)           www.cog-genomics.org/plink/1.9/
(C) 2005-2023 Shaun Purcell, Christopher Chang   GNU General Public License v3
Logging to dummy_project/rawdata.log.
Options in effect:
  --file rawdata/raw-GWA-data
  --make-bed
  --out dummy_project/rawdata

16384 MB RAM detected; reserving 8192 MB for main workspace.
.ped scan complete (for binary autoconversion).
Performing single-pass .bed write (317503 variants, 2000 people).
--file: dummy_project/rawdata-temporary.bed +
dummy_project/rawdata-temporary.bim + dummy_project/rawdata-temporary.fam
written.
317503 variants loaded from .bim file.
2000 people (997 males, 1003 females) loaded from .fam.
2000 phenotype values loaded from .fam.
Using 1 thread (no multithreaded calculations invoked).
Before main variant filters, 2000 founders and 0 nonfounders present.
Calculating allele frequencies... done.
Warning: 11440 het. haploid genotypes present (see dummy_project/rawdata.hh );
many commands treat these as missing.
Total genotyping rate is 0.985682.
317503 variants and 2000 people pass filters and QC.
Among remaining phenotypes, 1023 are cases and 977 are controls.
--make-bed to dummy_project/rawdata.bed + dummy_project/rawdata.bim +
dummy_project/rawdata.fam ... done.
```

So, there are 317,503 variants included for 2,000 people, 997 males and 1,003 females. All of these individuals are 'founders'. There are 1,023 cases and 977 controls. The genotyping rate is about 98.6% which is pretty good. Lastly, there are 11,440 heterozygous haploid genotypes present.

> Question: Can you think off what the '11,440 heterozygous haploid genotypes present' represent?

4.2 Quality control

We are ready for some quality control and quality assurance, heavily inspired by Anderson *et al.* (Anderson C.A., 2010) and Laurie *et al.* (Laurie C.C., 2010).

In general, we should check out a couple of things regarding the data quality on two levels:

1) samples
2) variants

So, we will investigate the following:

- Are the *sexes* based on genetic data matching the ones given by the phenotype file?
- Identify individuals that are outliers in terms of missing data (*call rate*) or heterozygosity rates. This could indicate a genotyping error or sample swap.
- Identify duplicated or related individuals.
- Identify individuals with divergent ancestry.
- What are the allele frequencies?
- What is the per-SNP call rate?
- In the case of a case-control study (which is the case here), we need to check differential missingness between cases and controls.

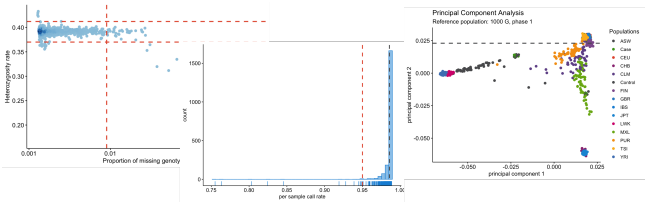
  By the way: you could extend the check on differential missingness to for instance 'genotyping platform', or 'hospital of inclusion', if you think this might influence the genotyping experiment technically.

4.3 Let's get our hands dirty

All clear? Let's start the work. On to step 1 of the QC for GWAS: filter samples of poor quality in Chapter 5.

Chapter 5

Sample QC



Let's start with the per-sample quality control.

5.1 Sex

We need to identify of individuals with discordant sex information comparing phenotypic and genotypic data. Let's calculate the mean homozygosity rate across X-chromosome markers for each individual in the study.

```
plink --bfile dummy_project/rawdata --check-sex --out dummy_project/rawdata
```

This produces a file with the following columns:

- *FID* Family ID
- *IID* Within-family ID
- *PEDSEX* Sex code in input file
- *SNPSEX* Imputed sex code (1 = male, 2 = female, 0 = unknown)
- *STATUS* 'OK' if PEDSEX and SNPSEX match and are nonzero, 'PROBLEM' otherwise
- *F* Inbreeding coefficient, considering only X chromosome. Not present with 'y-only'.

- *YCOUNT* Number of nonmissing genotype calls on Y chromosome. Requires 'ycount'/'y-only'.

We need to get a list of individuals with discordant sex data.

```
cat dummy_project/rawdata.sexcheck | awk '$5 =="STATUS" || $5 =="PROBLEM"'
> dummy_project/rawdata.sexprobs.txt
```

Let's have a look at the results.

```
cat dummy_project/rawdata.sexprobs.txt
```

```
library("data.table")

COURSE_loc = "~/Desktop/practical"  # getwd()

sexissues <- data.table::fread(paste0(COURSE_loc, "/dummy_project/rawdata.se

library("knitr")
knitr::kable(
  sexissues, caption = 'Sex issues',
  booktabs = TRUE
)
```

When the homozygosity rate (*F*) is more than 0.2, but less than 0.8, the genotype data are inconclusive regarding the sex of an individual and these are marked in column *SNPSEX* with a 0, and the column *STATUS* "PROBLEM".

Report the IDs of individuals with discordant sex information (Table **??**) to those who conducted sex phenotyping. In situations in which discrepancy cannot be resolved, add the family ID (FID) and individual ID (IID) of the samples to a file named fail –sexcheck–qc.txt (one individual per line, tab delimited).

```
grep "PROBLEM" dummy_project/rawdata.sexcheck | awk '{ print $1, $2}'
> dummy_project/fail–sexcheck–qc.txt
```

5.2 Sample call rates

Let's get an overview of the missing data per sample and per SNP.

```
plink --bfile dummy_project/rawdata --missing --out dummy_project/rawdata
```

This produces two files, rawdata/rawdata.imiss and rawdata/rawdata.lmiss. In the .imiss file the *N_MISS* column denotes the number of missing SNPs, and the *F_MISS* column denotes the proportion of missing SNPs per individual.

```
raw_IMISS <- data.table::fread(paste0(COURSE_loc, "/dummy_project/rawdata.imiss"))

raw_IMISS$callrate <- 1 - raw_IMISS$F_MISS
```

```
library("ggpubr")

ggpubr::gghistogram(raw_IMISS, x = "callrate", add = "mean",
  add.params = list(color = "#595A5C", linetype = "dashed",
    size = 1), rug = TRUE, bins = 50, color = "#1290D9",
  fill = "#1290D9", xlab = "per␣sample␣call␣rate") + ggplot2::geom_vline(xintercept =
  linetype = "dashed", color = "#E55738", size = 1)
ggplot2::ggsave(paste0(COURSE_loc, "/dummy_project/gwas-qc-sample-call-rate.png"),
  plot = last_plot())
```

The grey dashed line in Figure 5.1 indicates the mean call rate, while the red dashed line indicates the threshold we had determined above.

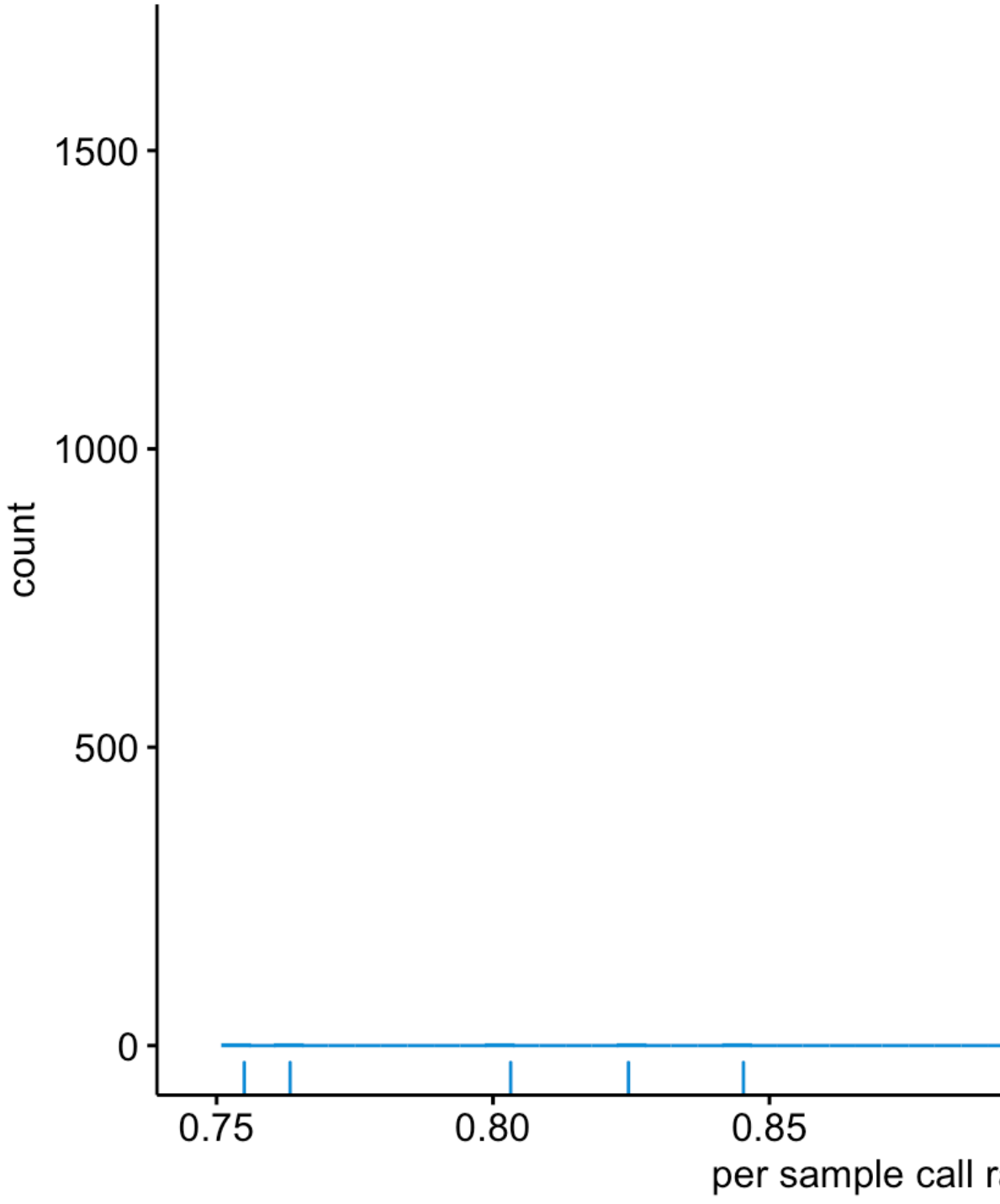


Figure 5.1: Per sample call rate.

5.3 Heterozygosity rate

To properly calculate heterozygosity rate and relatedness (identity-by-descent [IBD]) we need to do four things:

1) pre-clean the data to get a high-quality set,
2) of independent SNPs,
3) exclude long-range linkage disequilibrium (LD) blocks that bias with these calculations, and
4) exclude A/T and C/G SNPs as these may be ambivalent in interpretation when frequencies between cases and controls are close (MAF ± 0.45),
5) remove all non-autosomal SNPs.

You can find an up-to-date list of LD blocks you should exclude in these types of analyses here for the different genome builds. In this case we are using build 37. For the purpose of this book we included a file with these regions in the support-directory.

We will use the following settings:

- remove A/T and C/G SNPs with the flag –-exclude dummy_project/all.atcg.variants.txt,
- call rate <1% with the flag –-geno 0.10,
- Hardy-Weinberg Equilibrium (HWE) p-value > 1x10-3 with the flag –-hwe 1e–3,
- and MAF>10% with the flag –-maf 0.10,
- prune the data to only select independent SNPs (with low LD r^2) of one pair each with r^2 = 0.2 with the flags –-indep–pairwise 100 10 0.2 and –-extract rawdata/raw–GWA–data.prune.in,
- SNPs in long-range LD regions (for example: MHC chr 6 25.8-36Mb, chr 8 inversion 6-16Mb, chr17 40-45Mb, and a few more) with the flag –-exclude range support/exclude_problematic_range.txt,
- remove non-autosomal SNPs with the flag –-autosome.

First, get a list of A/T and C/G SNPs. Remember, the list of markers for this GWAS is noted in the .bim file. We can simply grep all the lines where the two alleles either have an A/T or C/G combination.

```
cat dummy_project/rawdata.bim | \
awk '($5 == "A" && $6 == "T") || ($5 == "T" && $6 == "A") || ($5 == "C" && $6 == "G")
> dummy_project/all.atcg.variants.txt
```

Second, clean the data and get a list of independent SNPs.

```
plink --bfile dummy_project/rawdata \
--autosome \
--maf 0.10 --geno 0.10 --hwe 1e-3 \
```

```
--indep-pairwise 100 10 0.2 \
--exclude range support/exclude_problematic_range.txt \
--make-bed --out dummy_project/rawdata.clean.temp
```

> Please note, we have create a dataset without taking into account LD structure. Hence, the message 'Pruned 0 variants from chromosome 1, leaving 19420.' etc. In a dataset without any LD structure this flag --indep-pairwise 100 10 0.2 doesn't actually work. However, with real-data you can use it to prune out unwanted SNPs in high LD.

Third, exclude the pruned SNPs. Note, how we include a file to exclude high-LD for the purpose of the practical.

```
plink --bfile dummy_project/rawdata.clean.temp \
--extract rawdata/raw-GWA-data.prune.in \
--make-bed --out dummy_project/rawdata.clean.ultraclean.temp
```

Fourth, remove the A/T and C/G SNPs.

```
plink --bfile dummy_project/rawdata.clean.ultraclean.temp \
--exclude dummy_project/all.atcg.variants.txt \
--make-bed --out dummy_project/rawdata.clean.ultraclean
```

> Please note, this dataset doesn't actually include this type of SNP, hence rawdata/all.atcg.variants.txt is empty! Again, you can use this command in real-data to exclude A/T and C/G SNPs.

Lastly, remove the temporary files.

```
rm -fv dummy_project/*.temp*
```

Finally, we can calculate the heterozygosity rate.

```
plink --bfile dummy_project/rawdata.clean.ultraclean --het --out dummy_proj
```

This creates the file dummy_project/rawdata.clean.ultraclean.het, in which the third column denotes the observed number of homozygous genotypes, O(Hom), and the fifth column denotes the number of nonmissing genotypes, N(NM), per individual. We can now calculate the observed heterozygosity rate per individual using the formula (N(NM) - O(Hom))/N(NM).

Often there is a correlation between heterozygosity rate and missing data. Thus, we should plot the observed heterozygosity rate per individual on the x-axis and the proportion of missing SNP, that is the 'SNP call rate', per individuals on the y-axis (Figure 5.2).

```
raw_HET <- data.table::fread(paste0(COURSE_loc, "/dummy_project/rawdata.clean.ultracl

raw_IMISS$logF_MISS = log10(raw_IMISS$F_MISS)
prop_miss = -1.522879

raw_HET$meanHet = (raw_HET$`N(NM)` - raw_HET$`O(HOM)`)/raw_HET$`N(NM)`
lower_meanHet = mean(raw_HET$meanHet) - (3 * sd(raw_HET$meanHet))
upper_meanHet = mean(raw_HET$meanHet) + (3 * sd(raw_HET$meanHet))

raw_IMISSHET = merge(raw_IMISS, raw_HET, by = "IID")
raw_IMISSHET$FID.y <- NULL
colnames(raw_IMISSHET)[colnames(raw_IMISSHET) == "FID.x"] <- "FID"

colors <- densCols(raw_IMISSHET$logF_MISS, raw_IMISSHET$meanHet)
```

```
library("geneplotter")
png(paste0(COURSE_loc, "/dummy_project/gwas-qc-imiss-vs-het.png"))
plot(raw_IMISSHET$logF_MISS, raw_IMISSHET$meanHet, col = colors,
  xlim = c(-3, 0), ylim = c(0, 0.5), pch = 20, xlab = "Proportion␣of␣missing␣genotype
  ylab = "Heterozygosity␣rate", axes = FALSE)
axis(2, at = c(0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35,
  0.4, 0.45, 0.5), tick = TRUE)
axis(1, at = c(-3, -2, -1, 0), labels = c(0.001, 0.01, 0.1,
  1))
abline(h = lower_meanHet, col = "#E55738", lty = 2)
abline(h = upper_meanHet, col = "#E55738", lty = 2)
abline(v = prop_miss, col = "#E55738", lty = 2)
```

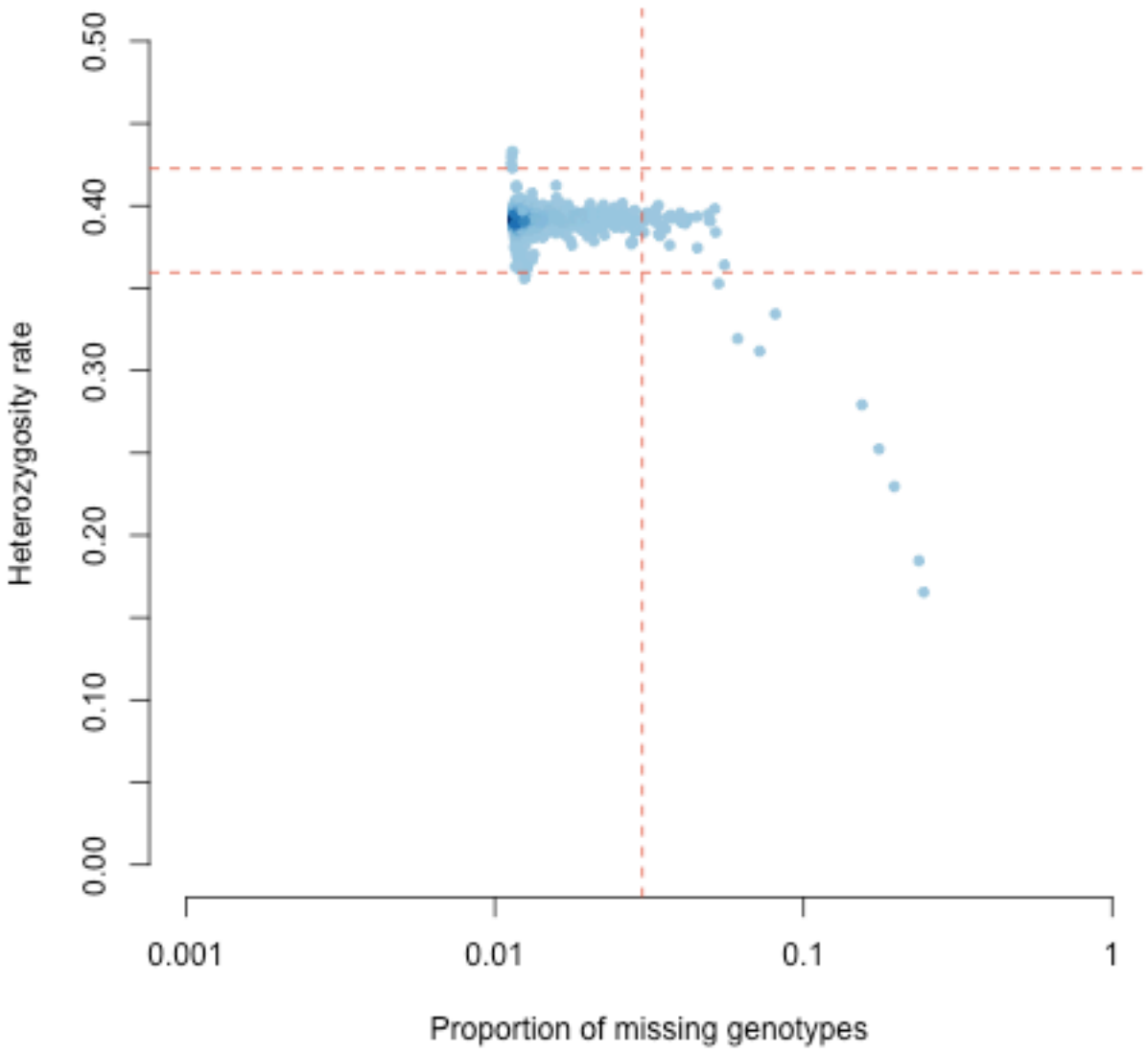


Figure 5.2: Heterozygosity as a function of SNP call rate.

Examine the plot (Figure 5.2) to decide reasonable thresholds at which to exclude individuals based on elevated missing or extreme heterozygosity. We

chose to exclude all individuals with a genotype failure rate >= 0.03 (vertical dashed line) and/or a heterozygosity rate ± 3 s.d. from the mean (horizontal dashed lines). Add the FID and IID of the samples failing this QC to the file named fail –imisshet–qc.txt.

How would you create this file?

```
raw_IMISSHETsub = subset(raw_IMISSHET, logF_MISS > prop_miss |
  (meanHet < lower_meanHet | meanHet > upper_meanHet),
  select = c("FID", "IID"))
data.table::fwrite(raw_IMISSHETsub, paste0(COURSE_loc, "/dummy_project/fail-raw_IMISS
  sep = "␣")
```

If all is right, you'd have something like Table **??**.

```
library("knitr")
knitr::kable(
  raw_IMISSHETsub, caption = 'Failed␣samples␣due␣to␣sample␣call␣rates␣and␣heterozygo
  booktabs = TRUE
)
```

5.4 Relatedness

We calculate Identity-by-Descent (IBS) to identify duplicated and related samples. In Table 5.4 we show how much DNA is shared between individuals depending on their relation(Stapples J., 2014). IBS is measured by calculating pi-hat ($\widehat{\pi}$), which is in essence the proportion of the DNA that a pair of samples share. To calculate this, we needed this ultraclean dataset, without low-quality SNPs and without high-LD regions.

\begin{table}

\caption{Familial relations and % DNA shared.}

Relatedness	%.DNA.sharing	IBD0	IBD1
Monozygotic twins	±100%	0	1
Parents/child	±50%	0.25	0.5
Sibling	±50%	0.25	0.5
Fraternal twins	±50%	0.25	0.5
Grandparent/grandchild	±25%	0.5	0.5
Aunt/Uncle/Niece/Nephew	±25%	0.5	0.5
Half-sibling	±25%	0.5	0.5
First-cousin	±12.5%	0.75	0.25
Half first-cousin	±6.25%	0.875	0.125
First-cousin once removed	±6.25%	0.875	0.125
Second-cousin	±3.13%	0.9375	6.25E-2
Second-cousin once removed	±1.56%	0.96875	3.125E-2
Distantly related	<1.56%	varies	varies
Unrelated (includes relationships beyond the third degree)	<1.56%	1	0

\end{table}

PLINK calculates the inter-individual relatedness using the –-genome function.

```
plink --bfile dummy_project/rawdata.clean.ultraclean --genome --out dummy_p
```

We can now identify all pairs of individuals with an IBD > 0.185. The code looks at the individual call rates stored in rawdata.imiss and outputs the IDs of the individual with the lowest call rate to 'fail-IBD-QC.txt' for subsequent removal (Table **??**).

First, move to the dummy_project directory.

```
cd dummy_project
```

Now, execute this script - it should work just fine out-of-the-box.

```
perl ../scripts/run-IBD-QC.pl rawdata rawdata.clean.ultraclean
```

Go back one directory.

```
cd ..
```

```
ibdcallissues <- data.table::fread(paste0(COURSE_loc, "/dummy_project/fail-I

knitr::kable(
  ibdcallissues,
  caption = 'Failed␣IBD␣and␣callrate.',
  # align = ,
  booktabs = FALSE
)
```

5.5 Ancestral background

Using a **Principal Component Analysis (PCA)** we can reduce the dimensions of the data, and project the "ancestral distances". In other words, the principal component 1 (the first dimension) and principal component 2 (the second dimension) which will capture most of the variation in the data and represent how much each sample is alike the next. And when compared to a reference, you can deduce the ancestral background of each sample in your dataset. Of course this is relative: we will only know that a given sample is very much a like samples from a given population that *exists today*.

Nowadays we run such PCA against a large and diverse dataset containing many different populations. Old-school GWAS (pre-2009) would compare a dataset against HapMap 3, nowadays we prefer at a minimum the 1000G phase 3 populations. And in those ancient times the preferred software to run a PCA was *Eigensoft* which is a bit tricky to install (see Chapter 9), but nowadays PLINK provides the –-pca-flag.

For the purpose of this practical primer we will run PCA against an earlier version of 1000G, phase 1, which is slightly smaller and just as good to use.

5.5.1 1000G phase 1

We will project our data to the reference, in this example 1000G phase 1 (1000G), which includes individuals from 14 distinct global populations across 4 'super'-populations (Europeans [EUR], Africans [AFR], East-Asians [EAS], and Latin Americans [AMR]). In the real-world, using phase 1 may be just fine, but if you think your population evolved through extensive migration it's probably best to use phase 3 data. In other words, the choice of reference is really depending on the dataset.

First, we will merge our data with 1000G. The alleles at each marker must be aligned to the same DNA strand to allow our data to merge correctly. Because not all SNPs are required for this analysis the A->T and C->G SNPs, which are more difficult to align, can be omitted.

5.5.1.1 Filter the 1000G data

First, we should get a list of relevant variants from our rawdata-dataset. We don't need the other variants present in the 1000G dataset, right?

```
cat dummy_project/rawdata.bim | grep "rs" > dummy_project/all.variants.txt
```

Extract those from the 1000G phase 1 data.

```
plink --bfile ref_1kg_phase1_all/1kg_phase1_all --extract dummy_project/all.variants.
```

5.5.1.2 Filter A/T & C/G SNPs

As explained, the A/T and C/G SNPs are problematic, we want to exclude these too. So let's get a list of A/T and C/G variants from 1000G to exclude - this may take a while.

```
cat ref_1kg_phase1_all/1kg_phase1_raw.bim | \
awk '($5 == "A" && $6 == "T") || ($5 == "T" && $6 == "A") || ($5 == "C" && $
> ref_1kg_phase1_all/all.1kg.atcg.variants.txt
```

Exclude those A/T and C/G variants in both datasets and at the same time filter to only retain high-quality data and exclude non-autosomal variants.

```
plink --bfile ref_1kg_phase1_all/1kg_phase1_raw --exclude ref_1kg_phase1_al

plink --bfile dummy_project/rawdata --exclude ref_1kg_phase1_all/all.1kg.atc
```

5.5.1.3 Merging datasets

Try and merge the data.

```
plink --bfile dummy_project/rawdata_1kg_phase1_raw_no_atcg --bmerge ref_1kg_
```

There probably is an error …

```
Error: 72 variants with 3+ alleles present.
* If you believe this is due to strand inconsistency, try --flip with
  dummy_project/rawdata.1kg_phase1-merge.missnp.
  (Warning: if the subsequent merge seems to work, strand errors involving S
  with A/T or C/G alleles probably remain in your data.
If LD between nearby
  SNPs is high, --flip-scan should detect them.)
* If you are dealing with genuine multiallelic variants, we recommend expor
  that subset of the data to VCF (via e.g. '--recode vcf'), merging with
  another tool/script, and then importing the result; PLINK is not yet suite
  to handling them.
See https://www.cog-genomics.org/plink/1.9/data#merge3 for more discussion.
```

So let's flip some variants.

```
plink --bfile dummy_project/rawdata --exclude ref_1kg_phase1_all/all.1kg.atc
```

Let's try again and merge the data.

```
plink --bfile dummy_project/rawdata_1kg_phase1_raw_no_atcg --bmerge ref_1kg_
```

There still is an error – there are a few multi-allelic variants present which PLINK can't handle.

```
Error: 14 variants with 3+ alleles present.
* If you believe this is due to strand inconsistency, try --flip with
  dummy_project/rawdata.1kg_phase1-merge.missnp.
  (Warning: if the subsequent merge seems to work, strand errors involving SNPs
  with A/T or C/G alleles probably remain in your data.
If LD between nearby
  SNPs is high, --flip-scan should detect them.)
* If you are dealing with genuine multiallelic variants, we recommend exporting
  that subset of the data to VCF (via e.g. '--recode vcf'), merging with
  another tool/script, and then importing the result; PLINK is not yet suited
  to handling them.
See https://www.cog-genomics.org/plink/1.9/data#merge3 for more discussion.
```

Let's just remove these multi-allelic variants.

```
plink --bfile dummy_project/rawdata_1kg_phase1_raw_no_atcg --exclude dummy_project/ra
```

After removing those pesky multi-allelic variants, we should be able to merge the data. We should take of the following:

- extract the pruned SNP-set (remember?), --extract rawdata/raw-GWA-data.prune.i,
- exclude non-autosomal variants, --autosome,
- and only keeping high-quality data, --maf 0.10 --geno 0.10 --hwe 1e-3,

```
plink --bfile dummy_project/rawdata_1kg_phase1_raw_no_atcg_bi \
--bmerge ref_1kg_phase1_all/1kg_phase1_raw_no_atcg \
--autosome \
--maf 0.10 --geno 0.10 --hwe 1e-3 \
--extract rawdata/raw-GWA-data.prune.in \
--make-bed --out dummy_project/rawdata.1kg_phase1.clean
```

Before we continue it's best to clean up a bit of the mess.

```
rm -fv dummy_project/rawdata_1kg_phase1_raw_no_atcg_bi* ref_1kg_phase1_all/1kg_phase
```

Now we have prepared our dataset with only high-quality SNPs that have few missing data, that are high-frequent, exclude problematic genomic ranges, and merged to the 1000G phase 1 reference dataset. Your output should look something like this:

```
PLINK v1.90b7.2 64-bit (11 Dec 2023)           www.cog-genomics.org/plink/1.9/
(C) 2005-2023 Shaun Purcell, Christopher Chang   GNU General Public License v3
Logging to dummy_project/rawdata.1kg_phase1.clean.log.
Options in effect:
  --autosome
  --bfile dummy_project/rawdata_1kg_phase1_raw_no_atcg_bi
  --bmerge ref_1kg_phase1_all/1kg_phase1_raw_no_atcg
```

```
  --extract rawdata/raw-GWA-data.prune.in
  --geno 0.10
  --hwe 1e-3
  --maf 0.10
  --make-bed
  --out dummy_project/rawdata.1kg_phase1.clean

16384 MB RAM detected; reserving 8192 MB for main workspace.
2000 people loaded from dummy_project/rawdata_1kg_phase1_raw_no_atcg_bi.fam.
1092 people to be merged from ref_1kg_phase1_all/1kg_phase1_raw_no_atcg.fam.
Of these, 1092 are new, while 0 are present in the base dataset.
Warning: Multiple positions seen for variant 'rs3934834'.
Warning: Multiple positions seen for variant 'rs3737728'.
Warning: Multiple positions seen for variant 'rs6687776'.
Warning: Multiple chromosomes seen for variant 'rs1050301'.
Warning: Multiple chromosomes seen for variant 'rs4850'.
317476 markers loaded from dummy_project/rawdata_1kg_phase1_raw_no_atcg_bi.b
312239 markers to be merged from ref_1kg_phase1_all/1kg_phase1_raw_no_atcg.b
Of these, 14 are new, while 312225 are present in the base dataset.
312190 more multiple-position warnings: see log file.
Performing single-pass merge (3092 people, 308317 variants).
Merged fileset written to dummy_project/rawdata.1kg_phase1.clean-merge.bed +
dummy_project/rawdata.1kg_phase1.clean-merge.bim +
dummy_project/rawdata.1kg_phase1.clean-merge.fam .
308317 variants loaded from .bim file.
3092 people (1522 males, 1570 females) loaded from .fam.
3092 phenotype values loaded from .fam.
--extract: 49856 variants remaining.
Using 1 thread (no multithreaded calculations invoked).
Before main variant filters, 3078 founders and 14 nonfounders present.
Calculating allele frequencies... done.
Total genotyping rate is 0.994867.
299 variants removed due to missing genotype data (--geno).
--hwe: 13825 variants removed due to Hardy-Weinberg exact test.
2617 variants removed due to minor allele threshold(s)
(--maf/--max-maf/--mac/--max-mac).
33115 variants and 3092 people pass filters and QC.
Phenotype data is quantitative.
--make-bed to dummy_project/rawdata.1kg_phase1.clean.bed +
dummy_project/rawdata.1kg_phase1.clean.bim +
dummy_project/rawdata.1kg_phase1.clean.fam ... done.
```

So in total there are 3,092 individuals, 1,522 males and 1,570 females, and 3,078 founders and 14 non-founders. The total genotyping rate is 99.5% and 33,115 variants are present.

5.5.2 Principal component analysis

Great, we've prepared our dummy project data and merged this with 1000G phase 1. Let's execute the PCA using --pca in PLINK.

```
plink --bfile dummy_project/rawdata.1kg_phase1.clean --pca --out dummy_project/rawdat
```

5.5.3 Plotting the PCA results

If all is peachy, you just succesfully ran PCA against 1000G phase 1. Using --pca we have calculated principal components (PCs), 20 in total by default, and we can now start plotting them. Let's create a scatter diagram of the first two principal components, including all individuals in the file rawdata.1kg_phase1.clean.eigenvec (the first and second principal components are columns 3 and 4, respectively). We need to collect some per-sample information to color the points according to sample origin.

Note: A R script for creating this plot (scripts/plot-pca-results.Rscript) is also provided (although any standard graphing software can be used), but below you'll find some fancy codes too.

First we collect the results from the --pca, the dummy data phenotype information, and the reference population information.

```
PCA_eigenval <- data.table::fread(paste0(COURSE_loc, "/dummy_project/rawdata.1kg_phas
PCA_eigenvec <- data.table::fread(paste0(COURSE_loc, "/dummy_project/rawdata.1kg_phas
ref_pop_raw <- data.table::fread(paste0(COURSE_loc, "/ref_1kg_phase1_all/1kg_phase1_a
dummy_pop <- data.table::fread(paste0(COURSE_loc, "/dummy_project/rawdata.fam"))

# Rename some
names(PCA_eigenval)[names(PCA_eigenval) == "V1"] <- "Eigenvalue"

names(PCA_eigenvec)[names(PCA_eigenvec) == "V1"] <- "FID"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V2"] <- "IID"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V3"] <- "PC1"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V4"] <- "PC2"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V5"] <- "PC3"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V6"] <- "PC4"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V7"] <- "PC5"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V8"] <- "PC6"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V9"] <- "PC7"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V10"] <- "PC8"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V11"] <- "PC9"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V12"] <- "PC10"
```

```
names(PCA_eigenvec)[names(PCA_eigenvec) == "V13"] <- "PC11"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V14"] <- "PC12"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V15"] <- "PC13"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V16"] <- "PC14"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V17"] <- "PC15"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V18"] <- "PC16"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V19"] <- "PC17"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V20"] <- "PC18"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V21"] <- "PC19"
names(PCA_eigenvec)[names(PCA_eigenvec) == "V22"] <- "PC20"

names(dummy_pop)[names(dummy_pop) == "V1"] <- "Family_ID"
names(dummy_pop)[names(dummy_pop) == "V2"] <- "Individual_ID"
names(dummy_pop)[names(dummy_pop) == "V5"] <- "Gender"
names(dummy_pop)[names(dummy_pop) == "V6"] <- "Phenotype"
dummy_pop$V3 <- NULL
dummy_pop$V4 <- NULL

dummy_pop$Population <- dummy_pop$Phenotype
dummy_pop$Population[dummy_pop$Population == 2] <- "Case"
dummy_pop$Population[dummy_pop$Population == 1] <- "Control"

# we subset the data we need
ref_pop <- subset(ref_pop_raw, select = c("Family_ID", "Individual_ID",
  "Gender", "Phenotype", "Population"))
rm(ref_pop_raw)

# we combine the reference and dummy information
ref_dummy_pop <- rbind(dummy_pop, ref_pop)

PCA_1kG <- merge(PCA_eigenvec, ref_dummy_pop, by.x = "IID",
  by.y = "Individual_ID", sort = FALSE, all.x = TRUE)

# Population    Description Super population    Code
Counts
# ASW   African Ancestry in Southwest US
AFR   4     #49A01D
# CEU   Utah residents with Northern and Western European ancestry
EUR 7     #E55738
# CHB   Han Chinese in Bejing, China
EAS   8     #9A3480
# CHS   Southern Han Chinese, China
EAS 9     #705296
# CLM   Colombian in Medellin, Colombia
MR  10  #8D5B9A
# FIN   Finnish in Finland
EUR 12  #2F8BC9
```

```
# GBR   British in England and Scotland
EUR 13  #1290D9
# IBS   Iberian populations in Spain
EUR   16  #1396D8
# JPT   Japanese in Tokyo, Japan
EAS   18  #D5267B
# LWK   Luhya in Webuye, Kenya
AFR 20  #78B113
# MXL   Mexican Ancestry in Los Angeles, California
AMR 22  #F59D10
# PUR   Puerto Rican in Puerto Rico
AMR 25  #FBB820
# TSI   Toscani in Italy
EUR   27  #4C81BF
# YRI   Yoruba in Ibadan, Nigeria
AFR   28  #C5D220

PCA_1kGplot <- ggpubr::ggscatter(PCA_1kG,
                                 x = "PC1",
                                 y = "PC2",
                                 color = "Population",
                                 palette = c("#49A01D", # ASW
                                             "#595A5C", # Case
                                             "#E55738", # CEU
                                             "#9A3480", # CHB
                                             "#705296", # CHS
                                             "#8D5B9A", # CLM
                                             "#A2A3A4", # Control
                                             "#2F8BC9", # FIN
                                             "#1290D9", # GBR
                                             "#1396D8", # IBS
                                             "#D5267B", # JPT
                                             "#78B113", # LWK
                                             "#F59D10", # MXL
                                             "#FBB820", # PUR
                                             "#4C81BF", # TSI
                                             "#C5D220"), # YRI
                                 xlab = "principal␣component␣1", ylab = "principal␣co
  ggplot2::geom_vline(xintercept = 0.0023, linetype = "dashed",
                      color = "#E55738", size = 1)

p2 <-  ggpubr::ggpar(PCA_1kGplot,
                     title = "Principal␣Component␣Analysis",
                     subtitle = "Reference␣population:␣1000␣G,␣phase␣1",
                     legend.title = "Populations", legend = "right")
ggplot2::ggsave(paste0(COURSE_loc, "/dummy_project/gwas-qc-pca-1000g.png"), plot = p2
```

```
p2
rm(p2)
```

Derive PC1 and PC2 thresholds so that only individuals who match the given ancestral population are included. For populations of European descent, this will be either the CEU or TSI 1000G individuals (Figure **??**). Here, we chose to exclude all individuals with a first principal component score less than 0.0023.

Write the FID and IID of these individuals to a file called fail–ancestry–QC.txt.

```
cat dummy_project/rawdata.1kg_phase1.clean.eigenvec | \
awk '$3 < 0.0023' | awk '{ print $1, $2 }' > dummy_project/fail-ancestry-QC.
```

Choosing which thresholds to apply (and thus which individuals to remove) is not a straightforward process. The key is to remove those individuals with greatly divergent ancestry, as these samples introduce the most bias to the study. Identification of more fine-scale ancestry can be conducted by using less divergent reference samples (*e.g.*, within Europe, stratification could be identified using the CEU, TSI (Italian), GBR (British), FIN (Finnish) and IBS (Iberian) samples from the 1,000 Genomes Project (http://www.1000genomes.org/)). Robust identification of fine-scale population structure often requires the construction of many (2–10) principal components.

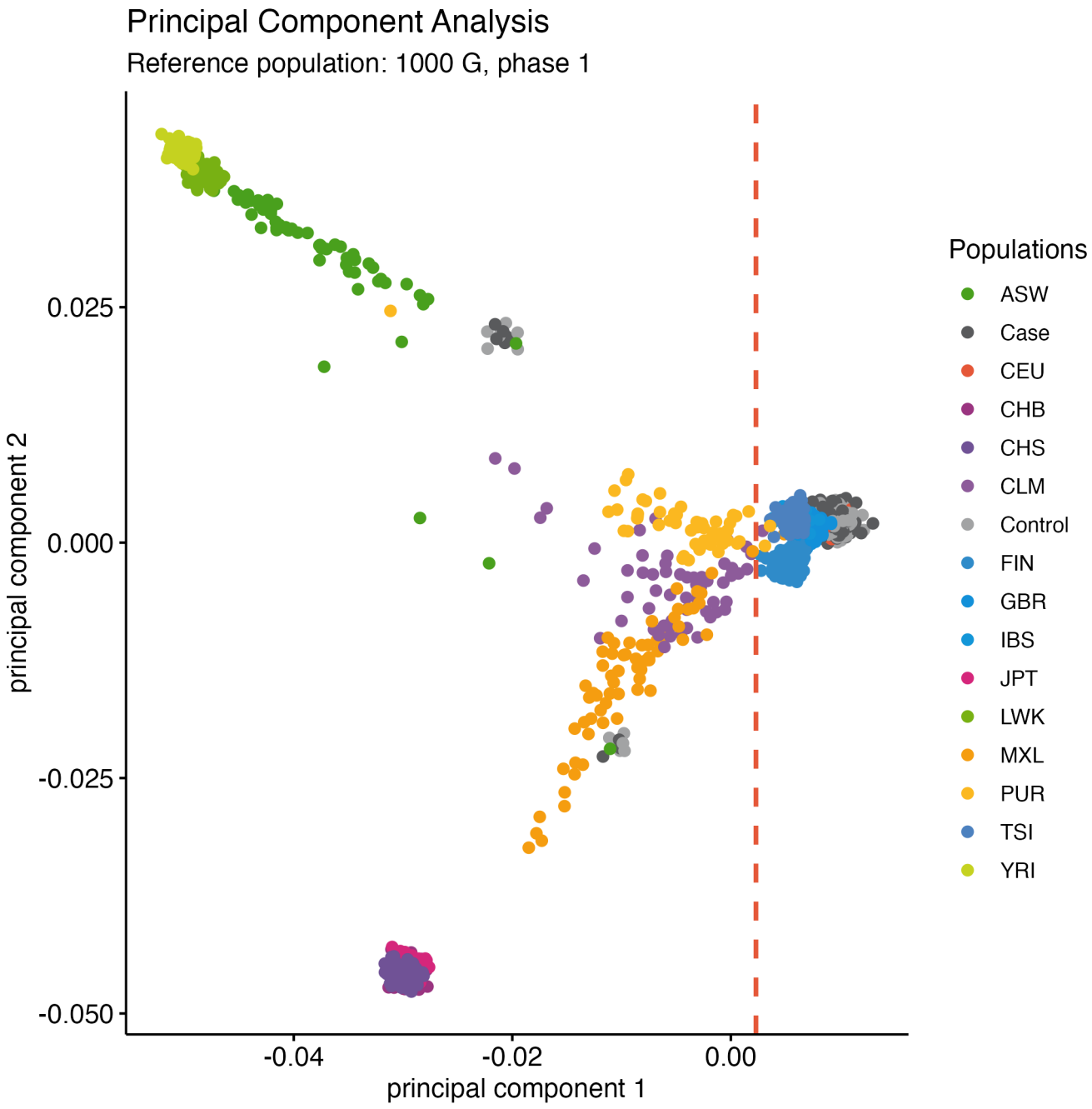


Figure 5.3: PCA - Your data vs. 1000g.

5.6 Removing samples

Finally! We have a list of samples of poor quality or divergent ancestry, and duplicated or related samples. We should remove these. Let's collect all IDs from our fail-*-files into a single file.

```
cat dummy_project/fail-* | sort -k1 | uniq > dummy_project/fail-qc-inds.txt
```

This new file should now contain a list of unique individuals failing the previous QC steps which we want to remove.

```
plink --bfile dummy_project/rawdata --remove dummy_project/fail-qc-inds.txt
```

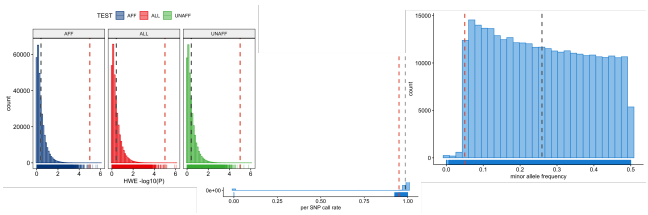
How many variants and samples are left? How many cases and how many controls did you loose?

5.7 The next step

Now that you filtered samples, we should turn our attention to step 2 of the QC for GWAS: identify SNPs of poor quality in Chapter @ref(gwas_basics_snp_qc).

Chapter 6

Per-SNP QC



Now that we removed samples, we can focus on low-quality variants.

6.1 SNP call rates

We start by calculating the missing genotype rate for each SNP, in other words the per-SNP call rate.

```
plink --bfile dummy_project/clean_inds_data --missing --out dummy_project/clean_inds_
```

Let's visualize the results to identify a threshold for extreme genotype failure rate. We chose a callrate threshold of 3%, but it's arbitrary and depending on the dataset and the number of samples (Figure 6.1).

```
library("data.table")

COURSE_loc = "~/Desktop/practical"  # getwd()

clean_LMISS <- data.table::fread(paste0(COURSE_loc, "/dummy_project/clean_inds_data.l

clean_LMISS$callrate <- 1 - clean_LMISS$F_MISS
```

```
library("ggpubr")

clean_LMISS_plot <- ggpubr::gghistogram(clean_LMISS, x = "callrate",
  add = "mean", add.params = list(color = "#595A5C", linetype = "dashed",
    size = 1), rug = TRUE, bins = 50, color = "#1290D9",
   fill = "#1290D9", xlab = "per␣SNP␣call␣rate") + ggplot2::geom_vline(xinter
  linetype = "dashed", color = "#E55738", size = 1)

ggplot2::ggsave(paste0(COURSE_loc, "/dummy_project/gwas-qc-snp-call-rate.png
  plot = clean_LMISS_plot)
clean_LMISS_plot
```

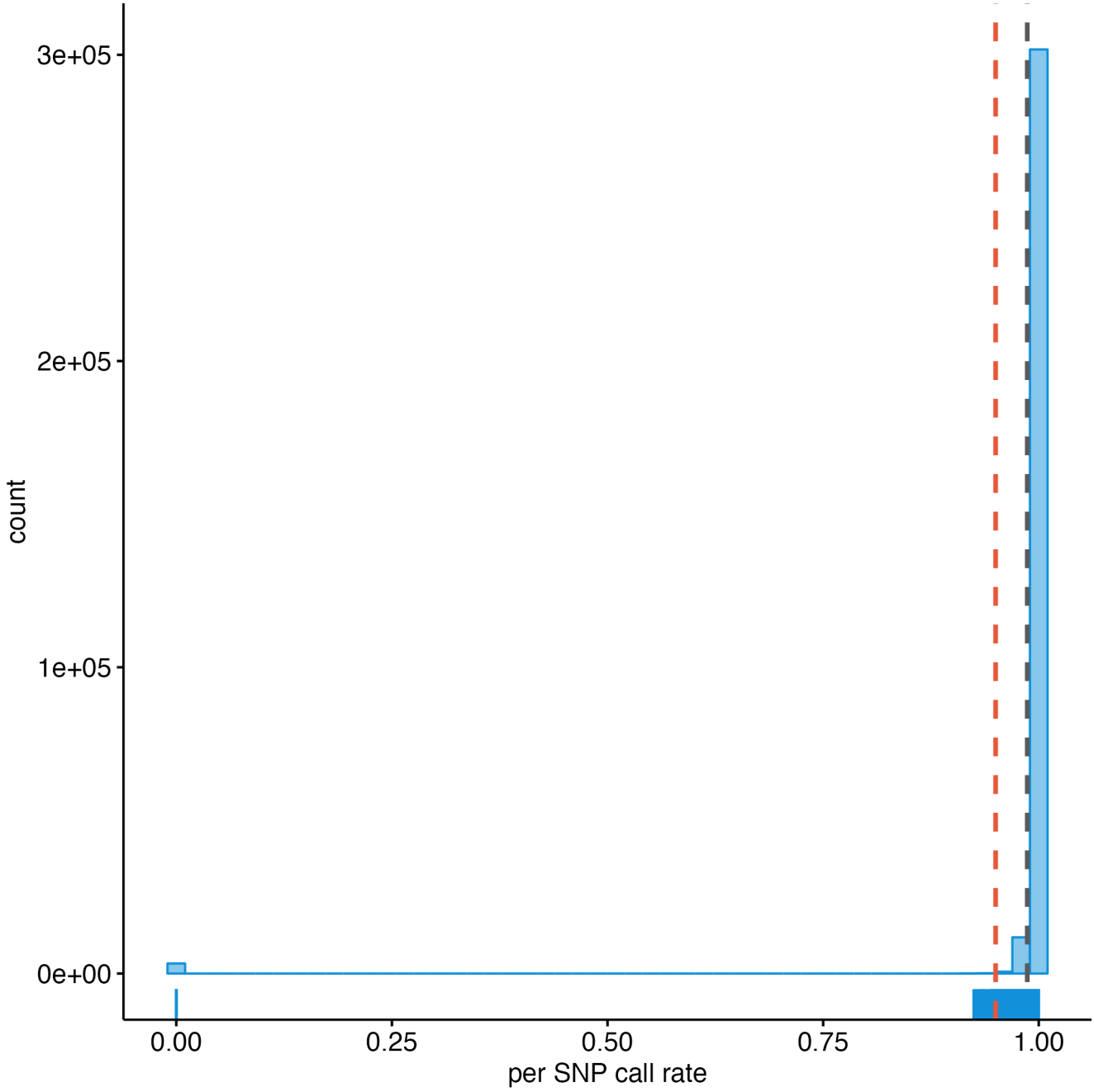


Figure 6.1: Per SNP call rate.

6.2 Differential SNP call rates

There could also be differences in genotype call rates between cases and controls. It is very important to check for this because these differences could lead to spurious associations. We can test all markers for differences in call rate between cases and controls, or based on

```
plink --bfile dummy_project/clean_inds_data --test-missing --out dummy_proj
```

Let's collect all the SNPs with a significantly different (P < 0.00001) missing data rate between cases and controls.

```
cat dummy_project/clean_inds_data.missing | awk '$5 < 0.00001' | awk '{ prin
```

6.3 Allele frequencies

We should also get an idea on what the allele frequencies are in our dataset. Low frequent SNPs should probably be excluded, as these are uninformative when monomorphic (allele frequency = 0), or they may lead to spurious associations.

```
plink --bfile dummy_project/clean_inds_data --freq --out dummy_project/clean
```

Let's also plot these data. You can view the result below, and try it yourself (Figure 6.2).

```
clean_FREQ <- data.table::fread(paste0(COURSE_loc, "/dummy_project/clean_inc
```

```
clean_FREQ_plot <- ggpubr::gghistogram(clean_FREQ, x = "MAF",
  add = "mean", add.params = list(color = "#595A5C", linetype = "dashed",
    size = 1), rug = TRUE, color = "#1290D9", fill = "#1290D9",
  xlab = "minor␣allele␣frequency") + ggplot2::geom_vline(xintercept = 0.05,
  linetype = "dashed", color = "#E55738", size = 1)

ggplot2::ggsave(paste0(COURSE_loc, "/dummy_project/gwas-qc-snp-freq.png"),
  plot = clean_FREQ_plot)
clean_FREQ_plot
```

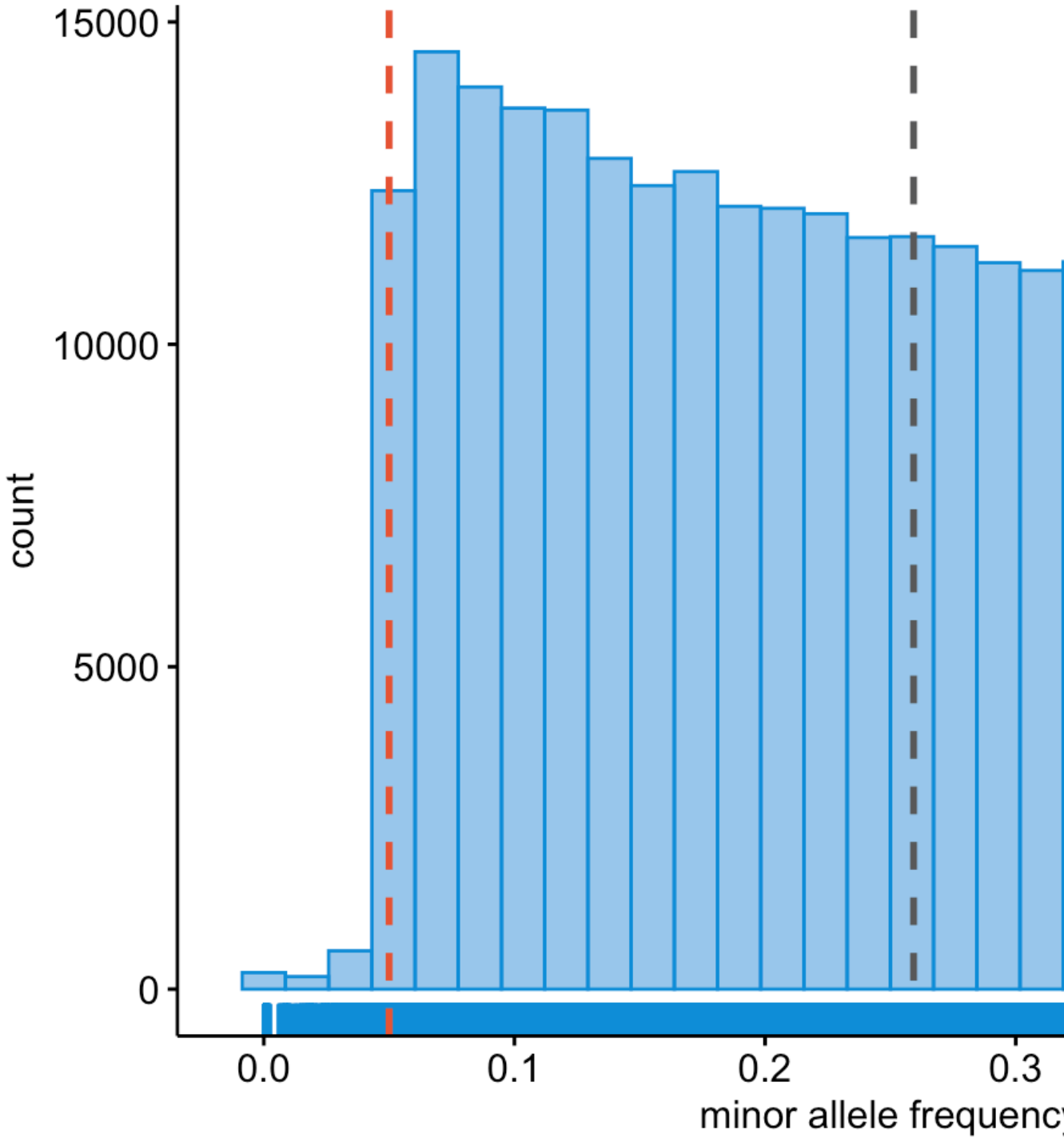


Figure 6.2: Minor allele frequency.

6.3.1 A note on allele coding

Oh, one more thing about alleles.

PLINK codes alleles as follows:

A1 = minor allele, the least frequent allele A2 = major allele, the most frequent allele

And when you use PLINK the flag –-freq or –-maf is always relative to the A1-allele, as is the odds ratio (OR) or effect size (beta).

However, SNPTEST makes use of the so-called OXFORD-format, this codes alleles as follows:

A = the 'other' allele B = the 'coded' allele

When you use SNPTEST it will report the allele frequency as CAF, in other words the *coded allele frequency*, and the effect size (beta) is always relative to the B-allele. This means, CAF *could* be the MAF, or *minor allele frequency*, but this is **not** a given.

In other words, always make sure what the allele-coding of a given program, be it PLINK, SNPTEST, GCTA, *et cetera*, is! I cannot stress this enough. Ask yourself: 'what is the allele frequency referring to?', 'the effect size is relative to…?'.

Right, let's continue.

6.4 Hardy-Weinberg Equilibrium

Because we are performing a case-control genome-wide association study, we probably expect some differences in Hardy-Weinberg Equilibrium (HWE), but extreme deviations are probably indicative of genotyping errors.

```
plink --bfile dummy_project/clean_inds_data --hardy --out dummy_project/clea
```

Let's also plot these data. You can view the result below, and type over the code to do it yourself.

```
clean_HWE <- data.table::fread(paste0(COURSE_loc, "/dummy_project/clean_inds
clean_HWE$logP <- -log10(clean_HWE$P)

clean_HWE_plot <- ggpubr::gghistogram(clean_HWE, x = "logP",
                                      add = "mean",
                                      add.params = list(color = "#595A5C", l
                                      rug = TRUE,
                                      # color = "#1290D9", fill = "#1290D9",
                                      color = "TEST", fill = "TEST",
```

```
                                    palette = "lancet",
                                    facet.by = "TEST",
                                    bins = 50,
                                    xlab = "HWE -log10(P)") +
  ggplot2::geom_vline(xintercept = 5, linetype = "dashed",
                      color = "#E55738", size = 1)

ggplot2::ggsave(paste0(COURSE_loc, "/dummy_project/gwas-qc-snp-hwe.png"), plot = clea
clean_HWE_plot
```

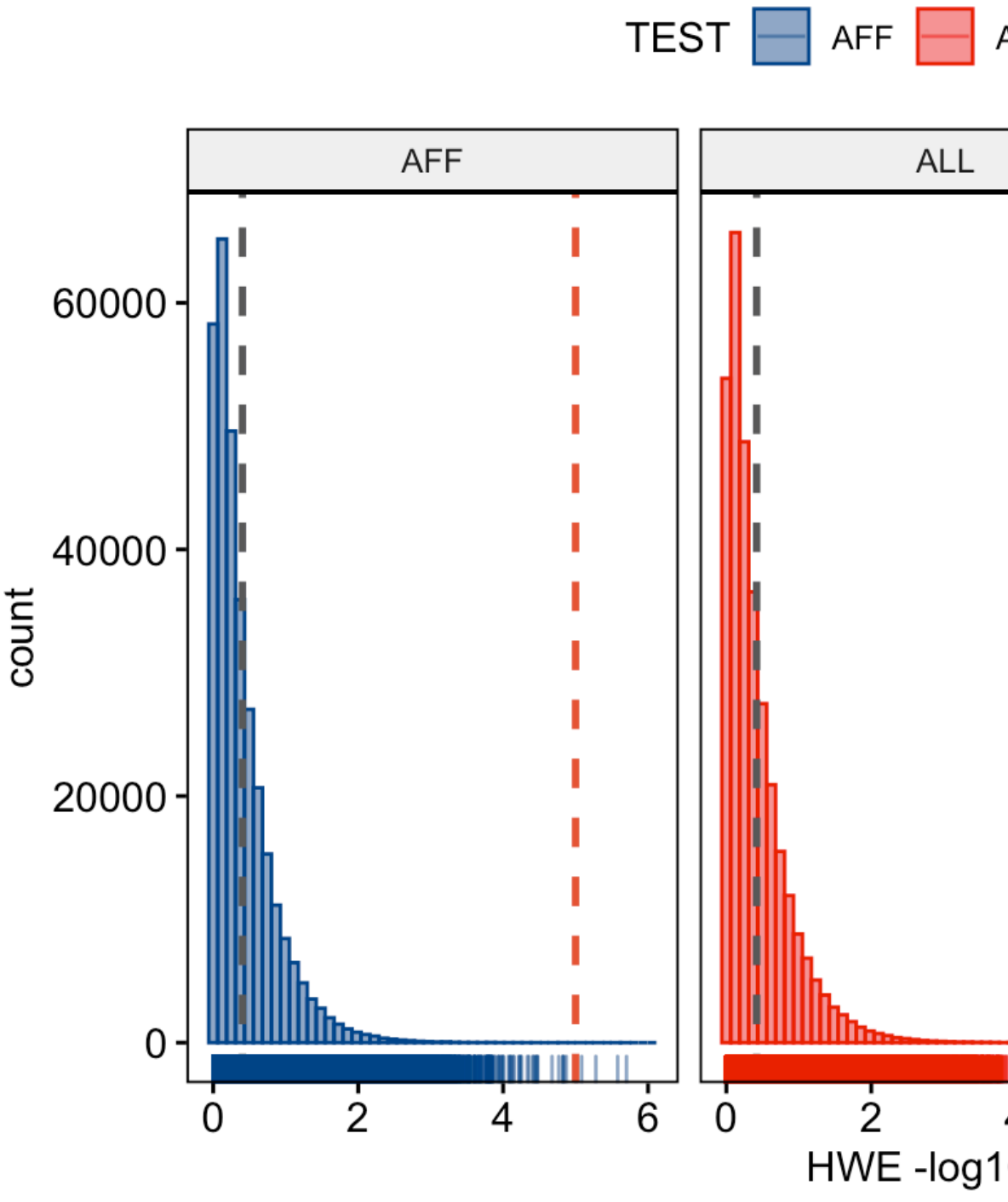


Figure 6.3: Hardy-Weinberg Equilibrium p-values per stratum.

6.5 Final SNP QC

We are ready to perform the final QC. After inspectig the graphs we will filter on a MAF < 0.01, call rate < 0.05, and HWE < 0.00001, in addition those SNPs that failed the differential call rate test will be removed.

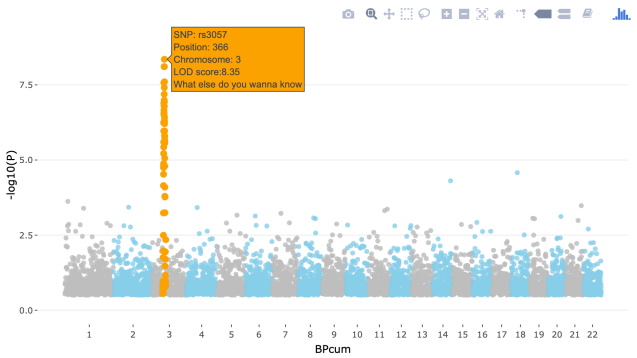
```
plink --bfile dummy_project/clean_inds_data --exclude dummy_project/fail-diffmiss-qc.
```

6.6 A Milestone

Congratulations. You reached a very important milestone. Now that you filtered samples and SNPs, we can finally start the association analyses in Chapter 7.

Chapter 7

Genome-Wide Association Study



Now that you have learned how to perform QC, you can easily run a GWAS and execute some downstream visualization and analyses. Let's do this with a dummy dataset.

7.1 Exploring the data

Even though someone says that the QC was done, it is still wise and good practice to run some of the commands above to get a 'feeling' about the data. So let's do this.

```
plink --bfile gwas/gwa --freq --out gwas/gwa

plink --bfile gwas/gwa --missing --out gwas/gwa
```

```
plink --bfile gwas/gwa --hardy --out gwas/gwa
```

Let's visualize the results. First we should load in all the results.

```
library("data.table")

COURSE_loc = "~/Desktop/practical"  # getwd()

gwas_HWE <- data.table::fread(paste0(COURSE_loc, "/gwas/gwa.hwe"))
gwas_FRQ <- data.table::fread(paste0(COURSE_loc, "/gwas/gwa.frq"))
gwas_IMISS <- data.table::fread(paste0(COURSE_loc, "/gwas/gwa.imiss"))
gwas_LMISS <- data.table::fread(paste0(COURSE_loc, "/gwas/gwa.lmiss"))
```

We can plot the per-stratum HWE p-values.

```
library("ggpubr")

gwas_HWE$logP <- -log10(gwas_HWE$P)

ggpubr::gghistogram(gwas_HWE, x = "logP",
                    add = "mean",
                    add.params = list(color = "#595A5C", linetype = "dashed'
                    rug = TRUE,
                    # color = "#1290D9", fill = "#1290D9",
                    color = "TEST", fill = "TEST",
                    palette = "lancet",
                    facet.by = "TEST",
                    bins = 50,
                    xlab = "HWE -log10(P)") +
  ggplot2::geom_vline(xintercept = 5, linetype = "dashed",
                      color = "#E55738", size = 1)
ggplot2::ggsave(paste0(COURSE_loc, "/dummy_project/gwas-hwe.png"),
       plot = last_plot())
```

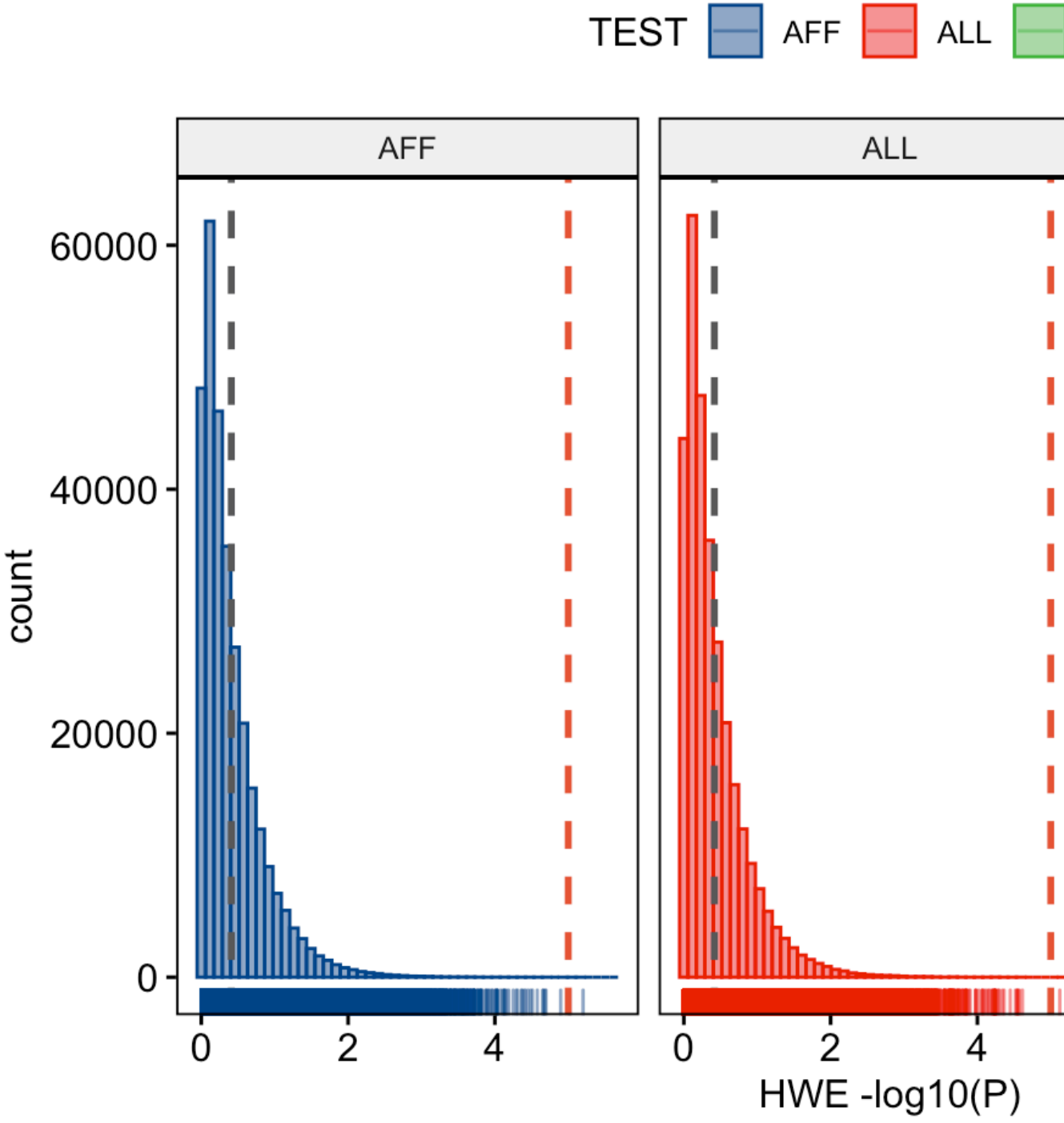


Figure 7.1: Per stratum HWE p-values.

We will want to see what the distribution of allele frequencies looks like.

```
ggpubr::gghistogram(gwas_FRQ, x = "MAF", add = "mean", add.params = list(co
  linetype = "dashed", size = 1), rug = TRUE, color = "#1290D9",
  fill = "#1290D9", xlab = "minor␣allele␣frequency") +
  ggplot2::geom_vline(xintercept = 0.05, linetype = "dashed",
    color = "#E55738", size = 1)
ggplot2::ggsave(paste0(COURSE_loc, "/dummy_project/gwas-freq.png"),
  plot = last_plot())
```

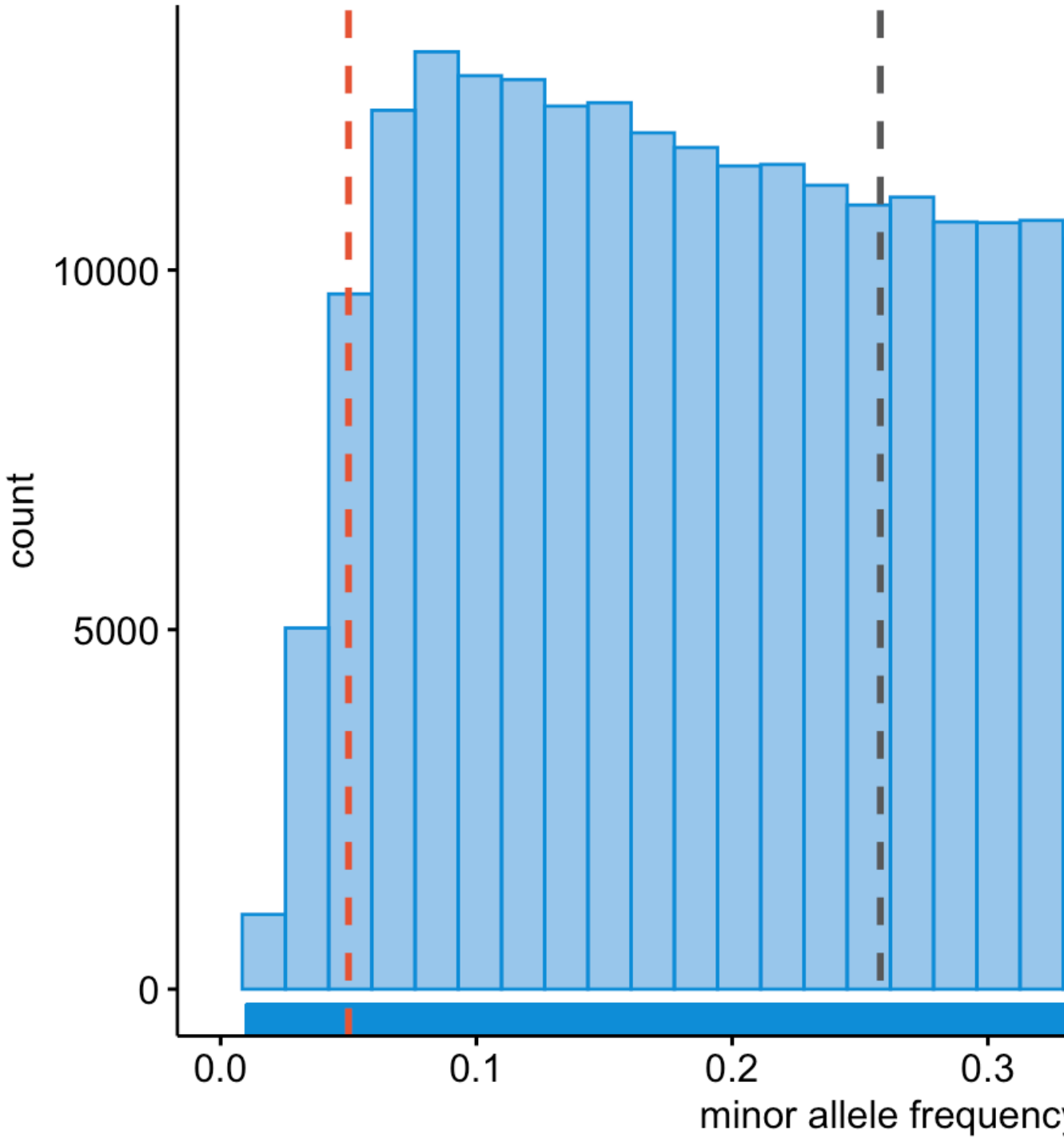


Figure 7.2: Minor allele frequencies.

We will want to identify samples that have poor call rates.

```
gwas_IMISS$callrate <- 1 - gwas_IMISS$F_MISS

ggpubr::gghistogram(gwas_IMISS, x = "callrate", add = "mean",
  add.params = list(color = "#595A5C", linetype = "dashed",
    size = 1), rug = TRUE, bins = 50, color = "#1290D9",
  fill = "#1290D9", xlab = "per␣sample␣call␣rate") + ggplot2::geom_vline(xi
  linetype = "dashed", color = "#E55738", size = 1)
ggplot2::ggsave(paste0(COURSE_loc, "/dummy_project/gwas-sample-call-rate.png
  plot = last_plot())
```

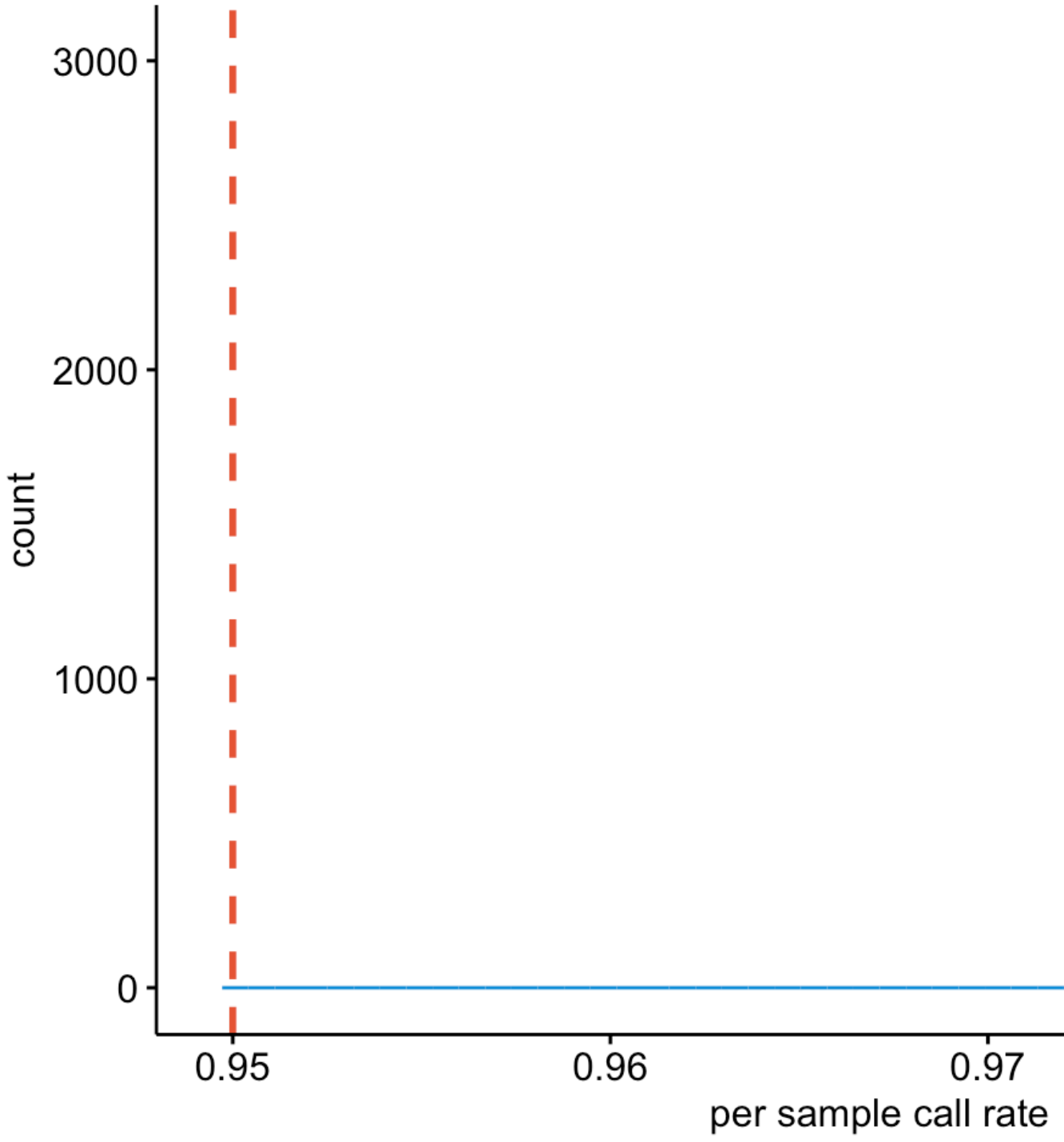


Figure 7.3: Per sample call rates.

We also need to know what the per SNP call rates are.

```
gwas_LMISS$callrate <- 1 - gwas_LMISS$F_MISS

ggpubr::gghistogram(gwas_LMISS, x = "callrate", add = "mean",
  add.params = list(color = "#595A5C", linetype = "dashed",
    size = 1), rug = TRUE, bins = 50, color = "#1290D9",
   fill = "#1290D9", xlab = "per␣SNP␣call␣rate") + ggplot2::geom_vline(xinter
  linetype = "dashed", color = "#E55738", size = 1)
ggplot2::ggsave(paste0(COURSE_loc, "/dummy_project/gwas-snp-call-rate.png"),
  plot = last_plot())
```

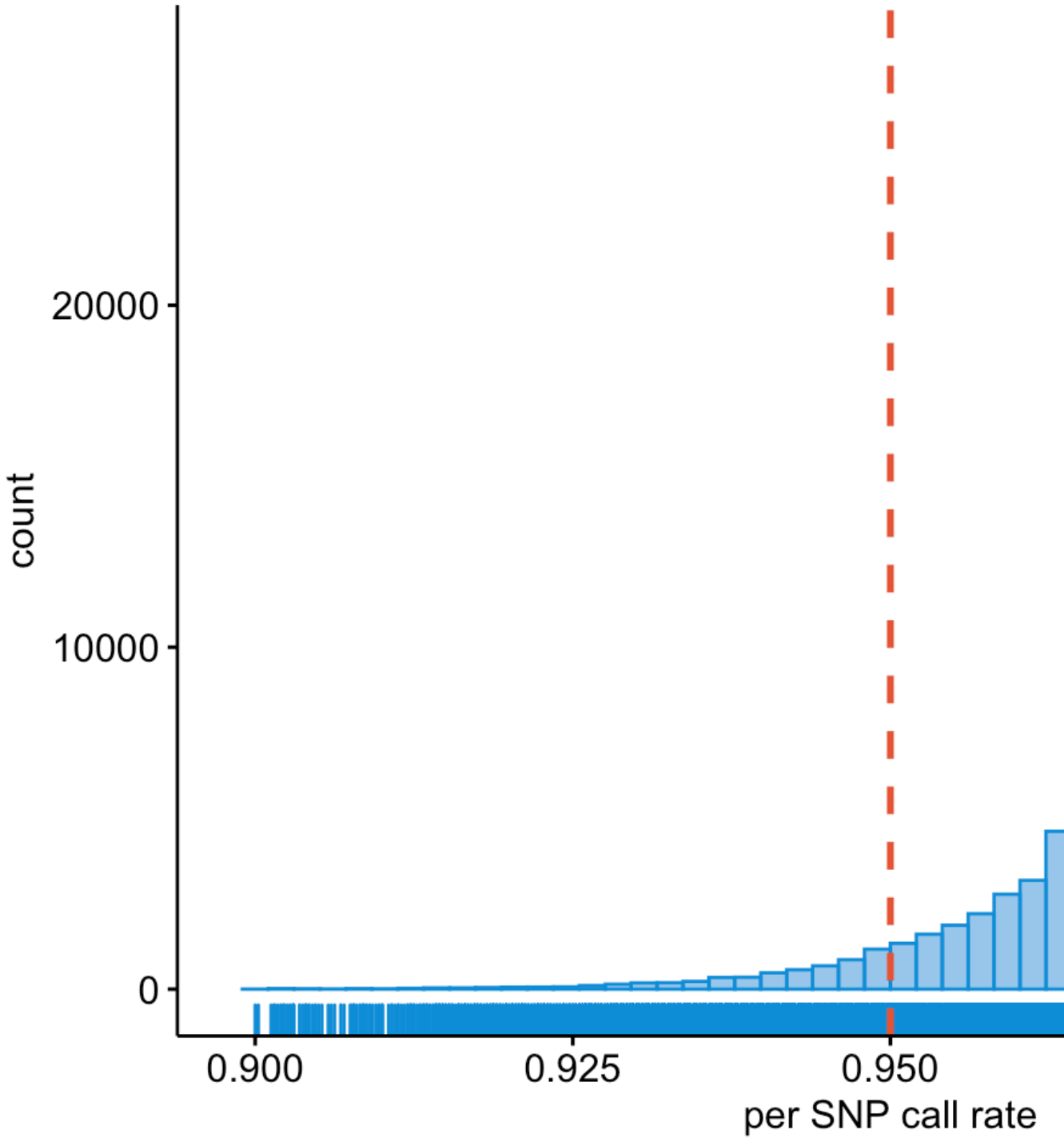


Figure 7.4: Per SNP call rates.

7.2 Genetic models

A simple chi-square test of association can be done.

```
plink --bfile gwas/gwa --model --out gwas/data
```

Genotypic, *dominant* and *recessive* tests will not be conducted if any one of the cells in the table of case-control by genotype counts contains less than five observations. This is because the chi-square approximation may not be reliable when cell counts are small. For SNPs with MAFs < 5%, a sample of more than 2,000 cases and controls would be required to meet this threshold and more than 50,000 would be required for SNPs with MAF < 1%.

You can change this default behaviour by adding the flag --cell, *e.g.*, we could lower the threshold to 3.

```
plink --bfile gwas/gwa --model --cell 3 --out gwas/data
```

Let's review the contents of the results.

```
gwas_model <- data.table::fread(paste0(COURSE_loc, "/gwas/data.model"))

dim(gwas_model)

N_SNPS = length(gwas_model$SNP)

gwas_model[1:10, 1:10]
```

It contains 1,530,510 rows, one for each SNP, and each type of test (*genotypic*, *trend*, *allelic*, *dominant*, and *recessive*) and the following columns:

- chromosome [CHR],
- the SNP identifier [SNP],
- the minor allele [A1] (remember, PLINK always codes the A1-allele as the minor allele!),
- the major allele [A2],
- the test performed [TEST]:
  - GENO (genotypic association);
  - TREND (Cochran-Armitage trend);
  - ALLELIC (allelic as- sociation);
  - DOM (dominant model); and
  - REC (recessive model)],
- the cell frequency counts for cases [AFF],
- the cell frequency counts for controls [UNAFF],
- the chi-square test statistic [CHISQ],
- the degrees of freedom for the test [DF],
- and the asymptotic P value [P] of association.

7.3 Logistic regression

We can also perform a test of association using logistic regression. In this case we might want to correct for covariates/confounding factors, for example age, sex, ancestral background, i.e. principal components, and other study specific covariates (e.g. hospital of inclusion, genotyping centre etc.). In that case each of these P values is adjusted for the effect of the covariates.

When running a regression analysis, be it linear or logistic, PLINK assumes a multiplicative model. By default, when at least one male and one female is present, sex (male = 1, female = 0) is automatically added as a covariate on X chromosome SNPs, and nowhere else. The sex flag causes it to be added everywhere, while no–x–sex excludes it.

```
plink --bfile gwas/gwa --logistic sex --covar gwas/gwa.covar --out gwas/data
```

Let's examine the results

```
gwas_assoc <- data.table::fread(paste0(COURSE_loc, "/gwas/data.assoc.logistic"))

dim(gwas_assoc)

gwas_assoc[1:9, 1:9]
```

If no model option is specified, the first row for each SNP corresponds to results for a multiplicative test of association. The C >= 0 subsequent rows for each SNP correspond to separate tests of significance for each of the C covariates included in the regression model. We can remove the covariate-specific lines from the main report by adding the hide–covar flag.

The columns in the association results are: - the chromosome [CHR], - the SNP identifier [SNP], - the base-pair location [BP], - the minor allele [A1], - the test performed [TEST]: ADD (multiplicative model or genotypic model testing additivity), - GENO_2DF (genotypic model), - DOMDEV (genotypic model testing deviation from additivity), - DOM (dominant model), or - REC (recessive model)], - the number of missing individuals included [NMISS], - the OR relative to the A1, *i.e.* minor allele, - the coefficient z-statistic [STAT], and - the asymptotic P-value [P] of association.

We need to calculate the standard error and confidence interval from the z-statistic. We can modify the effect size (OR) to output the beta by adding the beta flag.

7.4 Let's get visual

Looking at numbers is important, but it won't give you a perfect overview. We should turn to visualizing our results in Chapter **??**.

Chapter 8

Epilogue

What started as a simple ‘let’s write a practical on how to do a GWAS’, escalated into this GitBook. My second book. My fifth child (as far as I know). I hope you found it to be useful and learned a bit.

That said, much can be improved and so don’t hesitate to contact me.

As with any proper epilogue I should not forget a heartfelt and honest thank you to the readers and users of this work. Gratitude also goes to my dear colleagues Charlotte Onland, Jessica van Setten, and Kristel van Eijk, and former colleague Sara Pulit who asked me back in 2017 to join the course as a lecturer. It has been a pleasure to work with and learn from you, and it has been a fun (and sometimes stressful) experience to teach this course.

Chapter 9

Additional: EIGENSOFT

There used to be a time that the preferred software **EIGENSOFT** for Principal Component Analysis (PCA) was **EIGENSOFT** For many, it still is. However, **EIGENSOFT** is a bit challenging to make it work to say the least. You need to install some programs, and this is not always straightforward.

So, here's the deal.

I will share the how-to for a macOS environment below in [EIGENSOFT] - this should work in a Linux environment too as macOS is UNIX-based. You can choose to try and make it work on your system (be it UNIX or macOS based) at home (or in the office).

However, I recommend that you use the ––pca function which is present in PLINK v1.9 and up. This means you should probably simply skip this section and jump straight to Chapter 5.

9.1 Install homebrew

You need to install brew, the missing package-manager and accompanying packages that Apple didn't provide.

```
/bin/bash -c "$(curl -fsSL https://raw.githubusercontent.com/Homebrew/install/HEAD/ir
```

Next, check that everything is in order.

```
brew doctor
```

9.2 Install missing packages

Right, now that you've done that, you're ready to install gsl and openblas.

```
brew install gsl
brew install openblas
```

You may also require llvm for **EIGENSOFT** to work.

```
brew install llvm
```

9.3 Installing EIGENSOFT

I am still sharing the code you'll need - you could try this on your system.

```
mkdir -v $HOME/git
cd $HOME/git
git clone https://github.com/DReichLab/EIG.git
cd EIG/src
make
make install
```

Chapter 10

Things to do

I provide a list of things to do, to improve, to alter, to edit or to add. Crazy ideas. Useful tips, tricks, or links.

Obviously this list is not exhaustive nor intended for practical use for anyone else but me.

10.1 MoSCoW

List of to-do's according to MoSCoW: *must have*, *should have*, *could have*, *would have*.

10.1.1 Contents

- ☒ M add in full installation instructions for UBUNTU
- [] M add in full installation instructions for macOS
- [] M conditional analysis
- [] M statistical finemapping
- ☒ M regional association plotting
- [] M colocalization with formal testing
- [] S meta-analysis with dummy data
  - S including stratified QQ plots
  - M HPC version with MetaGWASToolKit
  - S stand-alone version with METAL
- [] C GWASToolKit
- [] C PlaqView lookups

10.2 Book fixes

- ☒ M overall rendering too slow, paste in images as figure instead of on the fly generating
- ☒ M PDF is too large
  - [] S PDF is not formatted properly (text runs over)
  - [] C Different font type in PDF
  - [] S EPUB is not formatted properly (text runs over)
  - [] S different setup for the chapter Additional chapters (this as a Apendix)
- ☒ S fix the way the team is displayed
  - [] S fix images per header in EPUB
  - [] S fix book cover
    - https://www.designhill.com/design-blog/the-perfect-ebook-cover-size-guide-and-publishing-tips/
    - https://snappa.com/blog/ebook-cover-size/
    - https://kdp.amazon.com/en_US/help/topic/G200645690.

10.3 Useful links (for me mostly)

https://bookdown.org/yihui/rmarkdown-cookbook/unnumbered-sections.html

https://bookdown.org/yihui/bookdown/cross-references.html

https://pandoc.org/MANUAL.html#extension-header_attributes

Add an image above the title:

- https://stackoverflow.com/questions/62074546/add-image-before-bookdown-title

But it causes an issue, described here. So you better remove this chunk of code:

```
\‘‘‘{js, echo = FALSE}
title=document.getElementById('header');
title.innerHTML = '<img src="img/_headers/banner_man_standing_dna.png" alt='
\‘‘‘
```

Chapter 11

Licenses and disclaimers

11.1 Copyright

This book and all its material (“content”) is protected by copyright under Dutch Copyright laws and is the property of the author or the party credited as the provider of the content. You may not copy, reproduce, distribute, publish, display, perform, modify, create derivative works, transmit, or in any way exploit any such content, nor may you distribute any part of this content over

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11.3 Images used

I used images from Unsplash to brighten up the book. These are listed here in no particular order.

- papers_on_wall - https://unsplash.com/photos/open-book-lot-Oaqk7qqNh_c
- women_behind_macbook - https://unsplash.com/photos/woman-using-macbook-pro-with-person-in-white-top-bPVM4nOy0Rg
- woman_working_on_code - https://unsplash.com/photos/woman-wearing-black-t-shirt-holding-white-computer-keyboard-YK0HPwWDJ1I
- licenses - https://unsplash.com/photos/book-lot-on-black-wooden-shelf-zeH-ljawHtg

Chapter 12

Colofon

[Add in text on colofon]

Bibliography

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Laurie C.C., e. a. (2010). Quality control and quality assurance in genotypic data for genome-wide association studies.

Purcell S., e. a. (2007). Plink, a tool set for whole-genome association and population-based linkage analyses.

Stapples J., e. a. (2014). Primus: Rapid reconstruction of pedigrees from genome-wide estimates of identity by descent.