GenSpace-Pgx_day2

July 25, 2020

1 GenSpace Workshop for Personalized Medicine

1.0.1 Date: July 26th, 2020

1.0.2 Objective: How to calculate Polygenic Risk Scores (PRS) to understand SSRI response

1.0.3 Facilitator: Kumar Veerapen, PhD and Caitlin Cooney, CGC

Creation of this hands-on workshop material is thanks to the inspiration from materials provided by Laramie Duncan, PhD, and Hanyang Shen, MS, Stanford University.

Materials from this notebook is complementary from the lecture slides found here

1.1 Learning objectives

1) To learn basic commands in Jupyter notebooks
2) To understand the calculation of PRS
3) To determine association of PRS to a genetic trait of interest
4) To define statistical limitations of PRS
5) To figure out what is considered a best fit PRS model

1.2 What is Polygenic Risk Score (PRS)?

An area in genetics that has large traction is understanding polygenicity of genetic traits. In recent years genome-wide association studies (GWAS) have discovered thousands of genetic variants associated both with diseases (depression, heart disease) and complex traits (height). Complex traits are traits derived from multiple genes, and exhibit a large range of attributes.

Polygenic risk scores evaluate these variants to help provide a personalized and more accurate risk/prediction assessment such as depression and height. The term PRS is synonymous with Genomic Risk Scores (GRS), Risk Profile Scores (RPS), or simply genetic scores.

Despite being a trendy method in understanding genetic traits, these scores are far from perfect. The PRS that have been published thus far usually tend to explain less than 20% of a trait variance in complex traits.

There are strengths and weaknesses to utilize PRS which are covered in the lecture session of this hands-on workshop.

1.3 What are we doing?

This hand-on workshop will allow you to explore a conventional approach to computing PRS using a ubiquitous genetics tool called PLINK.

Problem statement: Scientifically, we know that approx 53-65% of patients with depression respond well to antidepressants.

Hypothesis: We hypothesize that this variability is potentially caused by genetic risk.

Scientific question/objective: Are individuals with higher depression PRS more responsive to selective serotonin reuptake inhibitors (SSRI) [a type of antidepressant]?

1.4 Step by step overview

1.4.1 First...

We will obtain a reference data that contains effect sizes needed for calculating PRS.

Effect size : a statistical concept that measures the strength of the relationship between two variables on a numeric scale.
Example : If we look at a population's height, on average men are taller than women. This difference between the height of men and the height of women is known as the effect size. If the effect size is high, there is a higher height difference between men and women. If the effect size is small, there is less height difference.

The effect sizes (or summary statistics) that we will be using were generated from a reference data published by David Howard and co in *Nature Neuroscience* (2019). Their study attempted to understand the genetic risk of major depressive disorder in a very large collection of depressed patients. The reference data from this paper will contain effect sizes that we will need to calculate PRS in our test data which is a modified version of the 1000 Genomes Project.

The 1000 Genomes Project ran between 2008 and 2015, creating the largest public catalogue of human variation and genotype data

In this project, variation means to look for polymorphisms (genetic variants with frequencies of at least 1% in the populations studied)

1.4.2 Second...

We will calculate the PRS to answer our scientific question (refer to previous cell).

Using the effect sizes from our reference data (Howard et al 2019), we will then calculate PRS from samples obtained from a modified version of the 1000 Genomes data on a binary trait called selective serotonin reuptake inhibitor (SSRI) response. An SSRI is a typical group of antidepressants prescribed to patients with depressive symptoms.

The trait that you are analysing was simulated where

1 =the individual did not show an improvement in their depressive symptoms (measured by depression scores)

2 = the individual showed an improvement in their depressive symptoms (measured by depression scores)

1.4.3 Third...

We will use the PRS calculation to find an association to the trait that we are testing – SSRI response.

Finally, we will then use a statistical programming tool called R in order to 1) analyse the PRS generated; and 2) visualize our PRS using plots.

In order to provide a little understanding of the code that we are running, we have included some details in the comment section of your workshop.

Let's start with finding out how to use Jupyter notebook in order to run your analysis.

1.5 Using Jupyter

1.5.1 Running cells

Evaluate cells using SHIFT + ENTER. Select the next cell and run it

[1]:
```
print('Hello, world')
```

```
[1] "Hello, world"
```

1.5.2 Modes

Jupyter has two modes, a **navigation mode** and an **editor mode**.

Navigation mode:

- BLUE cell borders
- `UP` / `DOWN` move between cells
- `ENTER` while a cell is selected will move to **editing mode**.
- Many letters are keyboard shortcuts! This is a common trap.

Editor mode:

- GREEN cell borders
- `UP` / `DOWN`/ move within cells before moving between cells.
- `ESC` will return to **navigation mode**.
- `SHIFT + ENTER` will evaluate a cell and return to **navigation mode**.

1.5.3 Cell types

There are several types of cells in Jupyter notebooks. The two you will see here are **Markdown** (text) and **Code**.

[2]:
```
# This is a code cell
my_variable = 5
```

This is a markdown cell, so even if something looks like code (as below), it won't get executed!

my_variable += 1

1.5.4 Tips and tricks

Keyboard shortcuts:

- `SHIFT + ENTER` to evaluate a cell
- `ESC` to return to navigation mode
- `y` to turn a markdown cell into code
- `m` to turn a code cell into markdown
- `a` to add a new cell **above** the currently selected cell
- `b` to add a new cell **below** the currently selected cell
- `d, d` (repeated) to delete the currently selected cell
- `TAB` to activate code completion

To try this out, create a new cell below this one using `b`, and print `my_variable` by starting with `print(my` and pressing `TAB`!

1.6 Set up R environment with packages that you will need

R is a statistical programming tool (that's free) is a tool that many analysts use in order to analyse their datasets. This is especially true in genetics where we are cheap with buying software and analytic tools. With R, you can set up your environment to get additional tools to run some of your analyses.

The common practice in R is to load all of your needed tools (called packages) at the beginning of your script, i.e. the next line.

`fmsb` will be used in order to calculate the variance explained by the PRS that you will be computing The first line here, downloads the package into your environment.

Let's load the package into your environment now

[3]:
```
library(fmsb)
```

When you want to look up a function in R, you can use a `?` sign

[ ]:
```

```

2 Let's get started!

2.1 Step 0: Exploration of your files and directories

Where are you?

[4]:
```
getwd()
```

'/Users/kumar/Dropbox (Partners HealthCare)/Teaching_GenSpace_Pharmacogenetics/genspace_prs/resources

What files are in your directory (a.k.a. folder)?

[5]:
```
list.files()
```

1. 'GenSpace-Pgx_day2.ipynb' 2. 'HELP' 3. 'MDD_2019_logOR_score' 4. 'MDD_2019_logORpVal' 5. 'MDD_2019_pvalue_score' 6. 'OUTCOME' 7. 'Pherandom.reduced_1000_Genomes.bed' 8. 'Pherandom.reduced_1000_Genomes.bim' 9. 'Pherandom.reduced_1000_Genomes.fam' 10. 'plink' 11. 'plink.log' 12. 'q.ranges.GWASsig_to_1' 13. 'QC' 14. 'Ranalysis_PRS.R'

These are the files that we will be using for the workshop!

What files are what?

2.1.1 PLINK files

For PLINK, we have 3 files that will be used which are `.bed`, `.bim`, and `.fam` files.

These are known as binary PLINK files where

.bed file File name: `Pherandom.reduced_1000_Genomes.bed`

contains the genetic information for each individual in 0 and 1s. This file would contain individual level data such as deidentified sample ID, family ID (if applicable), and genotypes at multiple positions in the genome.
This file cannot be viewed because it is a binary file.

.bim file File name: `Pherandom.reduced_1000_Genomes.bim`

If the bed file contains the individual ID, the `.bim` file contain that generalized information. Think of the `.bim` files as Google map, and the `.bed` file as a person on the map.

This file contains locations of the genetic information that was captured such as which chromosome the variants are on. It has 6 columns and the most important are the 1st, 2nd, 4th, 5th, and 6th:

(1) Chromosome number

(2) SNP name / ID (oftentimes and 'rs' number)

(3) Chromosome Position

(4) Reference / Wildtype Allele

(5) Variant Allele

Let's view it with

[6]:
```
bim.file <- read.table("Pherandom.reduced_1000_Genomes.bim", header=F, sep="\t")
head(bim.file)
```

V1	V2	V3	V4	V5	V6
1	rs11579015	0	1036959	C	T
1	rs12029885	0	1125348	C	T
1	rs113908945	0	1183858	T	G
1	rs7549601	0	1213224	A	T
1	rs3766176	0	1486903	C	T
1	rs4648729	0	1808769	C	T

.fam file File name: `Pherandom.reduced_1000_Genomes.fam`

contains the individual ID and family name (for this workshop, ALL individuals have been deindentified).

The 1st, 2nd, and 6th columns are the important ones for this workshop.

(1) Family ID = FID

(2) Individual ID = IID

(3) Trait identification.

Note that `1` means control and `2` means case, per PLINK conventions. The trait values here are randomly generated where :

`1` would indicate that the individual did not show an improvement in their depressive symptoms.

`2` would indicate that the individual showed an improvement in their depressive symptoms

Let's view this file

[7]:
```
fam.file <- read.table("Pherandom.reduced_1000_Genomes.fam", header=F, sep="")
head(fam.file)
```

V1	V2	V3	V4	V5	V6
HG00096	HG00096	0	0	0	2
HG00097	HG00097	0	0	0	2
HG00099	HG00099	0	0	0	2
HG00100	HG00100	0	0	0	2
HG00101	HG00101	0	0	0	1
HG00102	HG00102	0	0	0	2

2.1.2 Files used for scoring

The reference data that we are using for computing PRS are also here in this environment. A reminder the reference data used in this workshop is from David Howard and co on genetic associations in major depressive disorder published in 2019.

There are 3 files that we will need which are the

1) "Score File",

2) "p-value file", and

3) the "TRANCHE file"

The "Score file" File name: `MDD_2019_logOR_score`.

This file contains the effect sizes from David Howard's paper needed for our PRS calculation. There are 3 columns in this file which are SNP ID, Risk allele, and logOR (the effect size that we keep talking about).

Let's view it

[8]:
```
score.file <- read.table("MDD_2019_logOR_score", header=T, sep="")
head(score.file)
```

SNP	allele	OR
rs11579015	T	0.02620370
rs12029885	T	0.00970278
rs113908945	T	0.03649590
rs7549601	A	-0.00879859
rs3766176	T	-0.02839950
rs4648729	T	-0.00810274

The "p-value file" File name : `MDD_2019_pvalue_score`.

This file contains the p-values from David Howard's paper that were used to tell if an effect size was considered significant or not.

As you are aware, we will need effect sizes in order to calculate PRS. These effect sizes were considered significant if they have a p-value that are less than 5 x 10-8 (0.000000005) from David Howard's paper. This value is a typical value used in genome-wide association studies (GWAS). That p-value would imply that the variant identified was 5 x 10-6 (0.0000005)% a false positive. The usage of p-values is ubiquitous in all of statistics so that analysts do not go down rabbit holes of signals which were bad apples to begin with.

This file contains 2 columns: `SNP ID` and `p-value`

Let's view the file (Notice that there are p-values which are traditionally, insignificant)

[9]:
```
pval.file <- read.table("MDD_2019_pvalue_score", header=T, sep="")
head(pval.file)
```

SNP	pval
rs11579015	0.12570
rs12029885	0.56880
rs113908945	0.03714
rs7549601	0.63200
rs3766176	0.02987
rs4648729	0.45270

The "TRANCHE file" File name: `q.ranges.GWASsig_to_1`.

This file contains tranches / bin / groups of p-value thresholds that will be used to group effect sizes for PRS calculations. The most strict of all p-values in the tranche file is the GWAS significant tranche (`PSi`). This would set our program (PLINK) to calculate PRS using effect sizes that were all having a p-value that are lower than 5 x 10-8 or 0.000000005 (`PSi`). Tranches listed in the file range from the strictest p-value threshold to the most lenient which includes all the variants that were tested in David Howard's paper (p-value threshold of lower than 1.0 (all)).

Let's view the file:

[10]:
```
tranche.file <- read.table("q.ranges.GWASsig_to_1", header=F, sep="")
head(tranche.file)
```

V1	V2	V3
Psi	0	5e-08
Pe6	0	1e-06
pe4	0	1e-04
pe3	0	1e-03
pe2	0	1e-02
P05	0	5e-02

Note : In this analysis, we will be using **pe2** (0.001) for our downstream applications.

2.2 Step 1 : Calculating Principal Components

(Timing Check) *Note* : This step will be done if we have time in the workshop. Also this step has been done for you and saved in your directory, just in case we are short on time.

Before we can start our PRS computations, we would need to capture the variance contributed by the different ancestries in our 1000 Genomes data. With multiple different ancestries, this may confound (a.k.a. mess up) our downstream analysis because humans are different from each other. By calculating principal components, we can capture some of the intrinsic differences observed in the 1000 Genome samples.

NOTE : there are a few other steps that we would need to do to clean the data up that I have done for you which include pruning the data for variants for variants which are informative such as common variants etc.

The tool that we can use to compute principal components is PLINK version 1.90.

To run this:

[11]:
```
system("./plink --bfile QC/Pherandom.reduced_1000_Genome.QC --pca 20 --out␣
↪OUTCOME/PCA_20_for_1000_Genomes", intern=T)
```

1. 'PLINK v1.90b6.2 64-bit (12 Jun 2018) www.cog-genomics.org/plink/1.9/' 2. '(C) 2005-2018 Shaun Purcell, Christopher Chang GNU General Public License v3' 3. 'Logging to OUTCOME/PCA_20_for_1000_Genomes.log.' 4. 'Options in effect:' 5. ' –bfile QC/Pherandom.reduced_1000_Genome.QC' 6. ' –out OUTCOME/PCA_20_for_1000_Genomes' 7. ' –pca 20' 8. '' 9. '16384 MB RAM detected; reserving 8192 MB for main workspace.' 10. '88086 variants loaded from .bim file.' 11. '2504 people (0 males, 0 females, 2504 ambiguous) loaded from .fam.' 12. 'Ambiguous sex IDs written to OUTCOME/PCA_20_for_1000_Genomes.nosex .' 13. '2504 phenotype values loaded from .fam.' 14. 'Using up to 4 threads (change this with –threads).' 15. 'Before main variant filters, 2504 founders and 0 nonfounders present.' 16. 'Calculating allele frequencies... 0%\b\b1%\b\b2%\b\b3%\b\b4%\b\b5%\b\b6%\b\b7%\b\b8%\b\b9%\b\b10%\b\b\b11%\b\b\b12%\b\b\b done.' 17. '88086 variants and 2504 people pass filters and QC.' 18. 'Note: No phenotypes present.' 19. '\r60 markers complete.\r120 markers complete.\r180 markers complete.\r240 markers complete.\r300 markers complete.\r360 markers complete.\r420 markers complete.\r480 markers complete.\r540 markers complete.\r600 markers complete.\r660 markers complete.\r720 markers complete.\r780 markers complete.\r840 markers complete.\r900 markers complete.\r960 markers complete.\r1020 markers complete.\r1080 markers complete.\r1140 markers complete.\r1200 markers complete.\r1260 markers complete.\r1320 markers complete.\r1380 markers complete.\r1440 markers complete.\r1500 markers complete.\r1560 markers complete.\r1620 markers

complete.\r1680 markers complete.\r1740 markers complete.\r1800 markers complete.\r1860 markers complete.\r1920 markers complete.\r1980 markers complete.\r2040 markers complete.\r2100 markers complete.\r2160 markers complete.\r2220 markers complete.\r2280 markers complete.\r2340 markers complete.\r2400 markers complete.\r2460 markers complete.\r2520 markers complete.\r2580 markers complete.\r2640 markers complete.\r2700 markers complete.\r2760 markers complete.\r2820 markers complete.\r2880 markers complete.\r2940 markers complete.\r3000 markers complete.\r3060 markers complete.\r3120 markers complete.\r3180 markers complete.\r3240 markers complete.\r3300 markers complete.\r3360 markers complete.\r3420 markers complete.\r3480 markers complete.\r3540 markers complete.\r3600 markers complete.\r3660 markers complete.\r3720 markers complete.\r3780 markers complete.\r3840 markers complete.\r3900 markers complete.\r3960 markers complete.\r4020 markers complete.\r4080 markers complete.\r4140 markers complete.\r4200 markers complete.\r4260 markers complete.\r4320 markers complete.\r4380 markers complete.\r4440 markers complete.\r4500 markers complete.\r4560 markers complete.\r4620 markers complete.\r4680 markers complete.\r4740 markers complete.\r4800 markers complete.\r4860 markers complete.\r4920 markers complete.\r4980 markers complete.\r5040 markers complete.\r5100 markers complete.\r5160 markers complete.\r5220 markers complete.\r5280 markers complete.\r5340 markers complete.\r5400 markers complete.\r5460 markers complete.\r5520 markers complete.\r5580 markers complete.\r5640 markers complete.\r5700 markers complete.\r5760 markers complete.\r5820 markers complete.\r5880 markers complete.\r5940 markers complete.\r6000 markers complete.\r6060 markers complete.\r6120 markers complete.\r6180 markers complete.\r6240 markers complete.\r6300 markers complete.\r6360 markers complete.\r6420 markers complete.\r6480 markers complete.\r6540 markers complete.\r6600 markers complete.\r6660 markers complete.\r6720 markers complete.\r6780 markers complete.\r6840 markers complete.\r6900 markers complete.\r6960 markers complete.\r7020 markers complete.\r7080 markers complete.\r7140 markers complete.\r7200 markers complete.\r7260 markers complete.\r7320 markers complete.\r7380 markers complete.\r7440 markers complete.\r7500 markers complete.\r7560 markers complete.\r7620 markers complete.\r7680 markers complete.\r7740 markers complete.\r7800 markers complete.\r7860 markers complete.\r7920 markers complete.\r7980 markers complete.\r8040 markers complete.\r8100 markers complete.\r8160 markers complete.\r8220 markers complete.\r8280 markers complete.\r8340 markers complete.\r8400 markers complete.\r8460 markers complete.\r8520 markers complete.\r8580 markers complete.\r8640 markers complete.\r8700 markers complete.\r8760 markers complete.\r8820 markers complete.\r8880 markers complete.\r8940 markers complete.\r9000 markers complete.\r9060 markers complete.\r9120 markers complete.\r9180 markers complete.\r9240 markers complete.\r9300 markers complete.\r9360 markers complete.\r9420 markers complete.\r9480 markers complete.\r9540 markers complete.\r9600 markers complete.\r9660 markers complete.\r9720 markers complete.\r9780 markers complete.\r9840 markers complete.\r9900 markers complete.\r9960 markers complete.\r10020 markers complete.\r10080 markers complete.\r10140 markers complete.\r10200 markers complete.\r10260 markers complete.\r10320 markers complete.\r10380 markers complete.\r10440 markers complete.\r10500 markers complete.\r10560 markers complete.\r10620 markers complete.\r10680 markers complete.\r10740 markers complete.\r10800 markers complete.\r10860 markers complete.\r10920 markers complete.\r10980 markers complete.\r11040 markers complete.\r11100 markers complete.\r11160 markers complete.\r11220 markers complete.\r11280 markers complete.\r11340 markers complete.\r11400 markers complete.\r11460 markers complete.\r11520 markers complete.\r11580 markers complete.\r11640 markers complete.\r11700 markers complete.\r11760 markers complete.\r11820 markers complete.\r11880 markers complete.\r11940 markers complete.\r12000 markers complete.\r12060 markers complete.\r12120

markers complete.\r12180 markers complete.\r12240 markers complete.\r12300 markers complete.\r12360 markers complete.\r12420 markers complete.\r12480 markers complete.\r12540 markers complete.\r12600 markers complete.\r12660 markers complete.\r12720 markers complete.\r12780 markers complete.\r12840 markers complete.\r12900 markers complete.\r12960 markers complete.\r13020 markers complete.\r13080 markers complete.\r13140 markers complete.\r13200 markers complete.\r13260 markers complete.\r13320 markers complete.\r13380 markers complete.\r13440 markers complete.\r13500 markers complete.\r13560 markers complete.\r13620 markers complete.\r13680 markers complete.\r13740 markers complete.\r13800 markers complete.\r13860 markers complete.\r13920 markers complete.\r13980 markers complete.\r14040 markers complete.\r14100 markers complete.\r14160 markers complete.\r14220 markers complete.\r14280 markers complete.\r14340 markers complete.\r14400 markers complete.\r14460 markers complete.\r14520 markers complete.\r14580 markers complete.\r14640 markers complete.\r14700 markers complete.\r14760 markers complete.\r14820 markers complete.\r14880 markers complete.\r14940 markers complete.\r15000 markers complete.\r15060 markers complete.\r15120 markers complete.\r15180 markers complete.\r15240 markers complete.\r15300 markers complete.\r15360 markers complete.\r15420 markers complete.\r15480 markers complete.\r15540 markers complete.\r15600 markers complete.\r15660 markers complete.\r15720 markers complete.\r15780 markers complete.\r15840 markers complete.\r15900 markers complete.\r15960 markers complete.\r16020 markers complete.\r16080 markers complete.\r16140 markers complete.\r16200 markers complete.\r16260 markers complete.\r16320 markers complete.\r16380 markers complete.\r16440 markers complete.\r16500 markers complete.\r16560 markers complete.\r16620 markers complete.\r16680 markers complete.\r16740 markers complete.\r16800 markers complete.\r16860 markers complete.\r16920 markers complete.\r16980 markers complete.\r17040 markers complete.\r17100 markers complete.\r17160 markers complete.\r17220 markers complete.\r17280 markers complete.\r17340 markers complete.\r17400 markers complete.\r17460 markers complete.\r17520 markers complete.\r17580 markers complete.\r17640 markers complete.\r17700 markers complete.\r17760 markers complete.\r17820 markers complete.\r17880 markers complete.\r17940 markers complete.\r18000 markers complete.\r18060 markers complete.\r18120 markers complete.\r18180 markers complete.\r18240 markers complete.\r18300 markers complete.\r18360 markers complete.\r18420 markers complete.\r18480 markers complete.\r18540 markers complete.\r18600 markers complete.\r18660 markers complete.\r18720 markers complete.\r18780 markers complete.\r18840 markers complete.\r18900 markers complete.\r18960 markers complete.\r19020 markers complete.\r19080 markers complete.\r19140 markers complete.\r19200 markers complete.\r19260 markers complete.\r19320 markers complete.\r19380 markers complete.\r19440 markers complete.\r19500 markers complete.\r19560 markers complete.\r19620 markers complete.\r19680 markers complete.\r19740 markers complete.\r19800 markers complete.\r19860 markers complete.\r19920 markers complete.\r19980 markers complete.\r20040 markers complete.\r20100 markers complete.\r20160 markers complete.\r20220 markers complete.\r20280 markers complete.\r20340 markers complete.\r20400 markers complete.\r20460 markers complete.\r20520 markers complete.\r20580 markers complete.\r20640 markers complete.\r20700 markers complete.\r20760 markers complete.\r20820 markers complete.\r20880 markers complete.\r20940 markers complete.\r21000 markers complete.\r21060 markers complete.\r21120 markers complete.\r21180 markers complete.\r21240 markers complete.\r21300 markers complete.\r21360 markers complete.\r21420 markers complete.\r21480 markers complete.\r21540 markers complete.\r21600 markers complete.\r21660 markers complete.\r21720 markers complete.\r21780 markers complete.\r21840 markers complete.\r21900 markers complete.\r21960 markers complete.\r22020 markers complete.\r22080 markers complete.\r22140 markers complete.\r22200

markers complete.\r22260 markers complete.\r22320 markers complete.\r22380 markers complete.\r22440 markers complete.\r22500 markers complete.\r22560 markers complete.\r22620 markers complete.\r22680 markers complete.\r22740 markers complete.\r22800 markers complete.\r22860 markers complete.\r22920 markers complete.\r22980 markers complete.\r23040 markers complete.\r23100 markers complete.\r23160 markers complete.\r23220 markers complete.\r23280 markers complete.\r23340 markers complete.\r23400 markers complete.\r23460 markers complete.\r23520 markers complete.\r23580 markers complete.\r23640 markers complete.\r23700 markers complete.\r23760 markers complete.\r23820 markers complete.\r23880 markers complete.\r23940 markers complete.\r24000 markers complete.\r24060 markers complete.\r24120 markers complete.\r24180 markers complete.\r24240 markers complete.\r24300 markers complete.\r24360 markers complete.\r24420 markers complete.\r24480 markers complete.\r24540 markers complete.\r24600 markers complete.\r24660 markers complete.\r24720 markers complete.\r24780 markers complete.\r24840 markers complete.\r24900 markers complete.\r24960 markers complete.\r25020 markers complete.\r25080 markers complete.\r25140 markers complete.\r25200 markers complete.\r25260 markers complete.\r25320 markers complete.\r25380 markers complete.\r25440 markers complete.\r25500 markers complete.\r25560 markers complete.\r25620 markers complete.\r25680 markers complete.\r25740 markers complete.\r25800 markers complete.\r25860 markers complete.\r25920 markers complete.\r25980 markers complete.\r26040 markers complete.\r26100 markers complete.\r26160 markers complete.\r26220 markers complete.\r26280 markers complete.\r26340 markers complete.\r26400 markers complete.\r26460 markers complete.\r26520 markers complete.\r26580 markers complete.\r26640 markers complete.\r26700 markers complete.\r26760 markers complete.\r26820 markers complete.\r26880 markers complete.\r26940 markers complete.\r27000 markers complete.\r27060 markers complete.\r27120 markers complete.\r27180 markers complete.\r27240 markers complete.\r27300 markers complete.\r27360 markers complete.\r27420 markers complete.\r27480 markers complete.\r27540 markers complete.\r27600 markers complete.\r27660 markers complete.\r27720 markers complete.\r27780 markers complete.\r27840 markers complete.\r27900 markers complete.\r27960 markers complete.\r28020 markers complete.\r28080 markers complete.\r28140 markers complete.\r28200 markers complete.\r28260 markers complete.\r28320 markers complete.\r28380 markers complete.\r28440 markers complete.\r28500 markers complete.\r28560 markers complete.\r28620 markers complete.\r28680 markers complete.\r28740 markers complete.\r28800 markers complete.\r28860 markers complete.\r28920 markers complete.\r28980 markers complete.\r29040 markers complete.\r29100 markers complete.\r29160 markers complete.\r29220 markers complete.\r29280 markers complete.\r29340 markers complete.\r29400 markers complete.\r29460 markers complete.\r29520 markers complete.\r29580 markers complete.\r29640 markers complete.\r29700 markers complete.\r29760 markers complete.\r29820 markers complete.\r29880 markers complete.\r29940 markers complete.\r30000 markers complete.\r30060 markers complete.\r30120 markers complete.\r30180 markers complete.\r30240 markers complete.\r30300 markers complete.\r30360 markers complete.\r30420 markers complete.\r30480 markers complete.\r30540 markers complete.\r30600 markers complete.\r30660 markers complete.\r30720 markers complete.\r30780 markers complete.\r30840 markers complete.\r30900 markers complete.\r30960 markers complete.\r31020 markers complete.\r31080 markers complete.\r31140 markers complete.\r31200 markers complete.\r31260 markers complete.\r31320 markers complete.\r31380 markers complete.\r31440 markers complete.\r31500 markers complete.\r31560 markers complete.\r31620 markers complete.\r31680 markers complete.\r31740 markers complete.\r31800 markers complete.\r31860 markers complete.\r31920 markers complete.\r31980 markers complete.\r32040 markers complete.\r32100 markers complete.\r32160 markers complete.\r32220 markers complete.\r32280

markers complete.\r32340 markers complete.\r32400 markers complete.\r32460 markers complete.\r32520 markers complete.\r32580 markers complete.\r32640 markers complete.\r32700 markers complete.\r32760 markers complete.\r32820 markers complete.\r32880 markers complete.\r32940 markers complete.\r33000 markers complete.\r33060 markers complete.\r33120 markers complete.\r33180 markers complete.\r33240 markers complete.\r33300 markers complete.\r33360 markers complete.\r33420 markers complete.\r33480 markers complete.\r33540 markers complete.\r33600 markers complete.\r33660 markers complete.\r33720 markers complete.\r33780 markers complete.\r33840 markers complete.\r33900 markers complete.\r33960 markers complete.\r34020 markers complete.\r34080 markers complete.\r34140 markers complete.\r34200 markers complete.\r34260 markers complete.\r34320 markers complete.\r34380 markers complete.\r34440 markers complete.\r34500 markers complete.\r34560 markers complete.\r34620 markers complete.\r34680 markers complete.\r34740 markers complete.\r34800 markers complete.\r34860 markers complete.\r34920 markers complete.\r34980 markers complete.\r35040 markers complete.\r35100 markers complete.\r35160 markers complete.\r35220 markers complete.\r35280 markers complete.\r35340 markers complete.\r35400 markers complete.\r35460 markers complete.\r35520 markers complete.\r35580 markers complete.\r35640 markers complete.\r35700 markers complete.\r35760 markers complete.\r35820 markers complete.\r35880 markers complete.\r35940 markers complete.\r36000 markers complete.\r36060 markers complete.\r36120 markers complete.\r36180 markers complete.\r36240 markers complete.\r36300 markers complete.\r36360 markers complete.\r36420 markers complete.\r36480 markers complete.\r36540 markers complete.\r36600 markers complete.\r36660 markers complete.\r36720 markers complete.\r36780 markers complete.\r36840 markers complete.\r36900 markers complete.\r36960 markers complete.\r37020 markers complete.\r37080 markers complete.\r37140 markers complete.\r37200 markers complete.\r37260 markers complete.\r37320 markers complete.\r37380 markers complete.\r37440 markers complete.\r37500 markers complete.\r37560 markers complete.\r37620 markers complete.\r37680 markers complete.\r37740 markers complete.\r37800 markers complete.\r37860 markers complete.\r37920 markers complete.\r37980 markers complete.\r38040 markers complete.\r38100 markers complete.\r38160 markers complete.\r38220 markers complete.\r38280 markers complete.\r38340 markers complete.\r38400 markers complete.\r38460 markers complete.\r38520 markers complete.\r38580 markers complete.\r38640 markers complete.\r38700 markers complete.\r38760 markers complete.\r38820 markers complete.\r38880 markers complete.\r38940 markers complete.\r39000 markers complete.\r39060 markers complete.\r39120 markers complete.\r39180 markers complete.\r39240 markers complete.\r39300 markers complete.\r39360 markers complete.\r39420 markers complete.\r39480 markers complete.\r39540 markers complete.\r39600 markers complete.\r39660 markers complete.\r39720 markers complete.\r39780 markers complete.\r39840 markers complete.\r39900 markers complete.\r39960 markers complete.\r40020 markers complete.\r40080 markers complete.\r40140 markers complete.\r40200 markers complete.\r40260 markers complete.\r40320 markers complete.\r40380 markers complete.\r40440 markers complete.\r40500 markers complete.\r40560 markers complete.\r40620 markers complete.\r40680 markers complete.\r40740 markers complete.\r40800 markers complete.\r40860 markers complete.\r40920 markers complete.\r40980 markers complete.\r41040 markers complete.\r41100 markers complete.\r41160 markers complete.\r41220 markers complete.\r41280 markers complete.\r41340 markers complete.\r41400 markers complete.\r41460 markers complete.\r41520 markers complete.\r41580 markers complete.\r41640 markers complete.\r41700 markers complete.\r41760 markers complete.\r41820 markers complete.\r41880 markers complete.\r41940 markers complete.\r42000 markers complete.\r42060 markers complete.\r42120 markers complete.\r42180 markers complete.\r42240 markers complete.\r42300 markers complete.\r42360

markers complete.\r42420 markers complete.\r42480 markers complete.\r42540 markers complete.\r42600 markers complete.\r42660 markers complete.\r42720 markers complete.\r42780 markers complete.\r42840 markers complete.\r42900 markers complete.\r42960 markers complete.\r43020 markers complete.\r43080 markers complete.\r43140 markers complete.\r43200 markers complete.\r43260 markers complete.\r43320 markers complete.\r43380 markers complete.\r43440 markers complete.\r43500 markers complete.\r43560 markers complete.\r43620 markers complete.\r43680 markers complete.\r43740 markers complete.\r43800 markers complete.\r43860 markers complete.\r43920 markers complete.\r43980 markers complete.\r44040 markers complete.\r44100 markers complete.\r44160 markers complete.\r44220 markers complete.\r44280 markers complete.\r44340 markers complete.\r44400 markers complete.\r44460 markers complete.\r44520 markers complete.\r44580 markers complete.\r44640 markers complete.\r44700 markers complete.\r44760 markers complete.\r44820 markers complete.\r44880 markers complete.\r44940 markers complete.\r45000 markers complete.\r45060 markers complete.\r45120 markers complete.\r45180 markers complete.\r45240 markers complete.\r45300 markers complete.\r45360 markers complete.\r45420 markers complete.\r45480 markers complete.\r45540 markers complete.\r45600 markers complete.\r45660 markers complete.\r45720 markers complete.\r45780 markers complete.\r45840 markers complete.\r45900 markers complete.\r45960 markers complete.\r46020 markers complete.\r46080 markers complete.\r46140 markers complete.\r46200 markers complete.\r46260 markers complete.\r46320 markers complete.\r46380 markers complete.\r46440 markers complete.\r46500 markers complete.\r46560 markers complete.\r46620 markers complete.\r46680 markers complete.\r46740 markers complete.\r46800 markers complete.\r46860 markers complete.\r46920 markers complete.\r46980 markers complete.\r47040 markers complete.\r47100 markers complete.\r47160 markers complete.\r47220 markers complete.\r47280 markers complete.\r47340 markers complete.\r47400 markers complete.\r47460 markers complete.\r47520 markers complete.\r47580 markers complete.\r47640 markers complete.\r47700 markers complete.\r47760 markers complete.\r47820 markers complete.\r47880 markers complete.\r47940 markers complete.\r48000 markers complete.\r48060 markers complete.\r48120 markers complete.\r48180 markers complete.\r48240 markers complete.\r48300 markers complete.\r48360 markers complete.\r48420 markers complete.\r48480 markers complete.\r48540 markers complete.\r48600 markers complete.\r48660 markers complete.\r48720 markers complete.\r48780 markers complete.\r48840 markers complete.\r48900 markers complete.\r48960 markers complete.\r49020 markers complete.\r49080 markers complete.\r49140 markers complete.\r49200 markers complete.\r49260 markers complete.\r49320 markers complete.\r49380 markers complete.\r49440 markers complete.\r49500 markers complete.\r49560 markers complete.\r49620 markers complete.\r49680 markers complete.\r49740 markers complete.\r49800 markers complete.\r49860 markers complete.\r49920 markers complete.\r49980 markers complete.\r50040 markers complete.\r50100 markers complete.\r50160 markers complete.\r50220 markers complete.\r50280 markers complete.\r50340 markers complete.\r50400 markers complete.\r50460 markers complete.\r50520 markers complete.\r50580 markers complete.\r50640 markers complete.\r50700 markers complete.\r50760 markers complete.\r50820 markers complete.\r50880 markers complete.\r50940 markers complete.\r51000 markers complete.\r51060 markers complete.\r51120 markers complete.\r51180 markers complete.\r51240 markers complete.\r51300 markers complete.\r51360 markers complete.\r51420 markers complete.\r51480 markers complete.\r51540 markers complete.\r51600 markers complete.\r51660 markers complete.\r51720 markers complete.\r51780 markers complete.\r51840 markers complete.\r51900 markers complete.\r51960 markers complete.\r52020 markers complete.\r52080 markers complete.\r52140 markers complete.\r52200 markers complete.\r52260 markers complete.\r52320 markers complete.\r52380 markers complete.\r52440

markers complete.\r52500 markers complete.\r52560 markers complete.\r52620 markers complete.\r52680 markers complete.\r52740 markers complete.\r52800 markers complete.\r52860 markers complete.\r52920 markers complete.\r52980 markers complete.\r53040 markers complete.\r53100 markers complete.\r53160 markers complete.\r53220 markers complete.\r53280 markers complete.\r53340 markers complete.\r53400 markers complete.\r53460 markers complete.\r53520 markers complete.\r53580 markers complete.\r53640 markers complete.\r53700 markers complete.\r53760 markers complete.\r53820 markers complete.\r53880 markers complete.\r53940 markers complete.\r54000 markers complete.\r54060 markers complete.\r54120 markers complete.\r54180 markers complete.\r54240 markers complete.\r54300 markers complete.\r54360 markers complete.\r54420 markers complete.\r54480 markers complete.\r54540 markers complete.\r54600 markers complete.\r54660 markers complete.\r54720 markers complete.\r54780 markers complete.\r54840 markers complete.\r54900 markers complete.\r54960 markers complete.\r55020 markers complete.\r55080 markers complete.\r55140 markers complete.\r55200 markers complete.\r55260 markers complete.\r55320 markers complete.\r55380 markers complete.\r55440 markers complete.\r55500 markers complete.\r55560 markers complete.\r55620 markers complete.\r55680 markers complete.\r55740 markers complete.\r55800 markers complete.\r55860 markers complete.\r55920 markers complete.\r55980 markers complete.\r56040 markers complete.\r56100 markers complete.\r56160 markers complete.\r56220 markers complete.\r56280 markers complete.\r56340 markers complete.\r56400 markers complete.\r56460 markers complete.\r56520 markers complete.\r56580 markers complete.\r56640 markers complete.\r56700 markers complete.\r56760 markers complete.\r56820 markers complete.\r56880 markers complete.\r56940 markers complete.\r57000 markers complete.\r57060 markers complete.\r57120 markers complete.\r57180 markers complete.\r57240 markers complete.\r57300 markers complete.\r57360 markers complete.\r57420 markers complete.\r57480 markers complete.\r57540 markers complete.\r57600 markers complete.\r57660 markers complete.\r57720 markers complete.\r57780 markers complete.\r57840 markers complete.\r57900 markers complete.\r57960 markers complete.\r58020 markers complete.\r58080 markers complete.\r58140 markers complete.\r58200 markers complete.\r58260 markers complete.\r58320 markers complete.\r58380 markers complete.\r58440 markers complete.\r58500 markers complete.\r58560 markers complete.\r58620 markers complete.\r58680 markers complete.\r58740 markers complete.\r58800 markers complete.\r58860 markers complete.\r58920 markers complete.\r58980 markers complete.\r59040 markers complete.\r59100 markers complete.\r59160 markers complete.\r59220 markers complete.\r59280 markers complete.\r59340 markers complete.\r59400 markers complete.\r59460 markers complete.\r59520 markers complete.\r59580 markers complete.\r59640 markers complete.\r59700 markers complete.\r59760 markers complete.\r59820 markers complete.\r59880 markers complete.\r59940 markers complete.\r60000 markers complete.\r60060 markers complete.\r60120 markers complete.\r60180 markers complete.\r60240 markers complete.\r60300 markers complete.\r60360 markers complete.\r60420 markers complete.\r60480 markers complete.\r60540 markers complete.\r60600 markers complete.\r60660 markers complete.\r60720 markers complete.\r60780 markers complete.\r60840 markers complete.\r60900 markers complete.\r60960 markers complete.\r61020 markers complete.\r61080 markers complete.\r61140 markers complete.\r61200 markers complete.\r61260 markers complete.\r61320 markers complete.\r61380 markers complete.\r61440 markers complete.\r61500 markers complete.\r61560 markers complete.\r61620 markers complete.\r61680 markers complete.\r61740 markers complete.\r61800 markers complete.\r61860 markers complete.\r61920 markers complete.\r61980 markers complete.\r62040 markers complete.\r62100 markers complete.\r62160 markers complete.\r62220 markers complete.\r62280 markers complete.\r62340 markers complete.\r62400 markers complete.\r62460 markers complete.\r62520

markers complete.\r62580 markers complete.\r62640 markers complete.\r62700 markers complete.\r62760 markers complete.\r62820 markers complete.\r62880 markers complete.\r62940 markers complete.\r63000 markers complete.\r63060 markers complete.\r63120 markers complete.\r63180 markers complete.\r63240 markers complete.\r63300 markers complete.\r63360 markers complete.\r63420 markers complete.\r63480 markers complete.\r63540 markers complete.\r63600 markers complete.\r63660 markers complete.\r63720 markers complete.\r63780 markers complete.\r63840 markers complete.\r63900 markers complete.\r63960 markers complete.\r64020 markers complete.\r64080 markers complete.\r64140 markers complete.\r64200 markers complete.\r64260 markers complete.\r64320 markers complete.\r64380 markers complete.\r64440 markers complete.\r64500 markers complete.\r64560 markers complete.\r64620 markers complete.\r64680 markers complete.\r64740 markers complete.\r64800 markers complete.\r64860 markers complete.\r64920 markers complete.\r64980 markers complete.\r65040 markers complete.\r65100 markers complete.\r65160 markers complete.\r65220 markers complete.\r65280 markers complete.\r65340 markers complete.\r65400 markers complete.\r65460 markers complete.\r65520 markers complete.\r65580 markers complete.\r65640 markers complete.\r65700 markers complete.\r65760 markers complete.\r65820 markers complete.\r65880 markers complete.\r65940 markers complete.\r66000 markers complete.\r66060 markers complete.\r66120 markers complete.\r66180 markers complete.\r66240 markers complete.\r66300 markers complete.\r66360 markers complete.\r66420 markers complete.\r66480 markers complete.\r66540 markers complete.\r66600 markers complete.\r66660 markers complete.\r66720 markers complete.\r66780 markers complete.\r66840 markers complete.\r66900 markers complete.\r66960 markers complete.\r67020 markers complete.\r67080 markers complete.\r67140 markers complete.\r67200 markers complete.\r67260 markers complete.\r67320 markers complete.\r67380 markers complete.\r67440 markers complete.\r67500 markers complete.\r67560 markers complete.\r67620 markers complete.\r67680 markers complete.\r67740 markers complete.\r67800 markers complete.\r67860 markers complete.\r67920 markers complete.\r67980 markers complete.\r68040 markers complete.\r68100 markers complete.\r68160 markers complete.\r68220 markers complete.\r68280 markers complete.\r68340 markers complete.\r68400 markers complete.\r68460 markers complete.\r68520 markers complete.\r68580 markers complete.\r68640 markers complete.\r68700 markers complete.\r68760 markers complete.\r68820 markers complete.\r68880 markers complete.\r68940 markers complete.\r69000 markers complete.\r69060 markers complete.\r69120 markers complete.\r69180 markers complete.\r69240 markers complete.\r69300 markers complete.\r69360 markers complete.\r69420 markers complete.\r69480 markers complete.\r69540 markers complete.\r69600 markers complete.\r69660 markers complete.\r69720 markers complete.\r69780 markers complete.\r69840 markers complete.\r69900 markers complete.\r69960 markers complete.\r70020 markers complete.\r70080 markers complete.\r70140 markers complete.\r70200 markers complete.\r70260 markers complete.\r70320 markers complete.\r70380 markers complete.\r70440 markers complete.\r70500 markers complete.\r70560 markers complete.\r70620 markers complete.\r70680 markers complete.\r70740 markers complete.\r70800 markers complete.\r70860 markers complete.\r70920 markers complete.\r70980 markers complete.\r71040 markers complete.\r71100 markers complete.\r71160 markers complete.\r71220 markers complete.\r71280 markers complete.\r71340 markers complete.\r71400 markers complete.\r71460 markers complete.\r71520 markers complete.\r71580 markers complete.\r71640 markers complete.\r71700 markers complete.\r71760 markers complete.\r71820 markers complete.\r71880 markers complete.\r71940 markers complete.\r72000 markers complete.\r72060 markers complete.\r72120 markers complete.\r72180 markers complete.\r72240 markers complete.\r72300 markers complete.\r72360 markers complete.\r72420 markers complete.\r72480 markers complete.\r72540 markers complete.\r72600

markers complete.\r72660 markers complete.\r72720 markers complete.\r72780 markers complete.\r72840 markers complete.\r72900 markers complete.\r72960 markers complete.\r73020 markers complete.\r73080 markers complete.\r73140 markers complete.\r73200 markers complete.\r73260 markers complete.\r73320 markers complete.\r73380 markers complete.\r73440 markers complete.\r73500 markers complete.\r73560 markers complete.\r73620 markers complete.\r73680 markers complete.\r73740 markers complete.\r73800 markers complete.\r73860 markers complete.\r73920 markers complete.\r73980 markers complete.\r74040 markers complete.\r74100 markers complete.\r74160 markers complete.\r74220 markers complete.\r74280 markers complete.\r74340 markers complete.\r74400 markers complete.\r74460 markers complete.\r74520 markers complete.\r74580 markers complete.\r74640 markers complete.\r74700 markers complete.\r74760 markers complete.\r74820 markers complete.\r74880 markers complete.\r74940 markers complete.\r75000 markers complete.\r75060 markers complete.\r75120 markers complete.\r75180 markers complete.\r75240 markers complete.\r75300 markers complete.\r75360 markers complete.\r75420 markers complete.\r75480 markers complete.\r75540 markers complete.\r75600 markers complete.\r75660 markers complete.\r75720 markers complete.\r75780 markers complete.\r75840 markers complete.\r75900 markers complete.\r75960 markers complete.\r76020 markers complete.\r76080 markers complete.\r76140 markers complete.\r76200 markers complete.\r76260 markers complete.\r76320 markers complete.\r76380 markers complete.\r76440 markers complete.\r76500 markers complete.\r76560 markers complete.\r76620 markers complete.\r76680 markers complete.\r76740 markers complete.\r76800 markers complete.\r76860 markers complete.\r76920 markers complete.\r76980 markers complete.\r77040 markers complete.\r77100 markers complete.\r77160 markers complete.\r77220 markers complete.\r77280 markers complete.\r77340 markers complete.\r77400 markers complete.\r77460 markers complete.\r77520 markers complete.\r77580 markers complete.\r77640 markers complete.\r77700 markers complete.\r77760 markers complete.\r77820 markers complete.\r77880 markers complete.\r77940 markers complete.\r78000 markers complete.\r78060 markers complete.\r78120 markers complete.\r78180 markers complete.\r78240 markers complete.\r78300 markers complete.\r78360 markers complete.\r78420 markers complete.\r78480 markers complete.\r78540 markers complete.\r78600 markers complete.\r78660 markers complete.\r78720 markers complete.\r78780 markers complete.\r78840 markers complete.\r78900 markers complete.\r78960 markers complete.\r79020 markers complete.\r79080 markers complete.\r79140 markers complete.\r79200 markers complete.\r79260 markers complete.\r79320 markers complete.\r79380 markers complete.\r79440 markers complete.\r79500 markers complete.\r79560 markers complete.\r79620 markers complete.\r79680 markers complete.\r79740 markers complete.\r79800 markers complete.\r79860 markers complete.\r79920 markers complete.\r79980 markers complete.\r80040 markers complete.\r80100 markers complete.\r80160 markers complete.\r80220 markers complete.\r80280 markers complete.\r80340 markers complete.\r80400 markers complete.\r80460 markers complete.\r80520 markers complete.\r80580 markers complete.\r80640 markers complete.\r80700 markers complete.\r80760 markers complete.\r80820 markers complete.\r80880 markers complete.\r80940 markers complete.\r81000 markers complete.\r81060 markers complete.\r81120 markers complete.\r81180 markers complete.\r81240 markers complete.\r81300 markers complete.\r81360 markers complete.\r81420 markers complete.\r81480 markers complete.\r81540 markers complete.\r81600 markers complete.\r81660 markers complete.\r81720 markers complete.\r81780 markers complete.\r81840 markers complete.\r81900 markers complete.\r81960 markers complete.\r82020 markers complete.\r82080 markers complete.\r82140 markers complete.\r82200 markers complete.\r82260 markers complete.\r82320 markers complete.\r82380 markers complete.\r82440 markers complete.\r82500 markers complete.\r82560 markers complete.\r82620 markers complete.\r82680

markers complete.\r82740 markers complete.\r82800 markers complete.\r82860 markers complete.\r82920 markers complete.\r82980 markers complete.\r83040 markers complete.\r83100 markers complete.\r83160 markers complete.\r83220 markers complete.\r83280 markers complete.\r83340 markers complete.\r83400 markers complete.\r83460 markers complete.\r83520 markers complete.\r83580 markers complete.\r83640 markers complete.\r83700 markers complete.\r83760 markers complete.\r83820 markers complete.\r83880 markers complete.\r83940 markers complete.\r84000 markers complete.\r84060 markers complete.\r84120 markers complete.\r84180 markers complete.\r84240 markers complete.\r84300 markers complete.\r84360 markers complete.\r84420 markers complete.\r84480 markers complete.\r84540 markers complete.\r84600 markers complete.\r84660 markers complete.\r84720 markers complete.\r84780 markers complete.\r84840 markers complete.\r84900 markers complete.\r84960 markers complete.\r85020 markers complete.\r85080 markers complete.\r85140 markers complete.\r85200 markers complete.\r85260 markers complete.\r85320 markers complete.\r85380 markers complete.\r85440 markers complete.\r85500 markers complete.\r85560 markers complete.\r85620 markers complete.\r85680 markers complete.\r85740 markers complete.\r85800 markers complete.\r85860 markers complete.\r85920 markers complete.\r85980 markers complete.\r86040 markers complete.\r86100 markers complete.\r86160 markers complete.\r86220 markers complete.\r86280 markers complete.\r86340 markers complete.\r86400 markers complete.\r86460 markers complete.\r86520 markers complete.\r86580 markers complete.\r86640 markers complete.\r86700 markers complete.\r86760 markers complete.\r86820 markers complete.\r86880 markers complete.\r86940 markers complete.\r87000 markers complete.\r87060 markers complete.\r87120 markers complete.\r87180 markers complete.\r87240 markers complete.\r87300 markers complete.\r87360 markers complete.\r87420 markers complete.\r87480 markers complete.\r87540 markers complete.\r87600 markers complete.\r87660 markers complete.\r87720 markers complete.\r87780 markers complete.\r87840 markers complete.\r87900 markers complete.\r87960 markers complete.\r88020 markers complete.\r88080 markers complete.\r88086 markers complete.\rRelationship matrix calculation complete.' 20. '[extracting eigenvalues and eigenvectors]\r–pca: Results saved to OUTCOME/PCA_20_for_1000_Genomes.eigenval and' 21. 'OUTCOME/PCA_20_for_1000_Genomes.eigenvec .'

`--bfile` is to call in the PLINK binary files that we will need. This contains the 1000 Genomes samples

`--pca` generate principal components and state number of components wanted, i.e. 20

`--out` is to print out the principal components

Where is our output PCs?

[12]:
```
list.files(path="OUTCOME", pattern="PCA_20_for_1000_Genomes")
```

1. 'PCA_20_for_1000_Genomes.eigenval' 2. 'PCA_20_for_1000_Genomes.eigenvec' 3. 'PCA_20_for_1000_Genomes.log' 4. 'PCA_20_for_1000_Genomes.nosex'

2.3 Step 2: Calculating Polygenic Risk Scores

We have understood what files contain what pieces of information/data that we would need for our final analysis.

We now get to compute PRS for our data. In order to do this, we can run the PLINK command as below, where description of each flag follows the code block

[13]:
```
system("./plink --bfile Pherandom.reduced_1000_Genome --score␣
↪MDD_2019_logORpVal 1 2 3 header no-mean-imputation --q-score-range q.ranges.
↪GWASsig_to_1 MDD_2019_pvalue_score --extract QC/Pherandom.
↪reduced_1000_Genome.QC.clumped.valid.snp --allow-no-sex --out OUTCOME/MDD",␣
↪intern=T)
```

```
Warning message in system("./plink --bfile Pherandom.reduced_1000_Genome --score
MDD_2019_logORpVal 1 2 3 header no-mean-imputation --q-score-range
q.ranges.GWASsig_to_1 MDD_2019_pvalue_score --extract
QC/Pherandom.reduced_1000_Genome.QC.clumped.valid.snp --allow-no-sex --out
OUTCOME/MDD", :
"running command './plink --bfile Pherandom.reduced_1000_Genome --score
MDD_2019_logORpVal 1 2 3 header no-mean-imputation --q-score-range
q.ranges.GWASsig_to_1 MDD_2019_pvalue_score --extract
QC/Pherandom.reduced_1000_Genome.QC.clumped.valid.snp --allow-no-sex --out
OUTCOME/MDD' had status 2"
```

1. 'PLINK v1.90b6.2 64-bit (12 Jun 2018) www.cog-genomics.org/plink/1.9/' 2. '(C) 2005-2018 Shaun Purcell, Christopher Chang GNU General Public License v3' 3. 'Logging to OUTCOME/MDD.log.' 4. 'Options in effect:' 5. ' –allow-no-sex' 6. ' –bfile Pherandom.reduced_1000_Genome' 7. ' –extract QC/Pherandom.reduced_1000_Genome.QC.clumped.valid.snp' 8. ' –out OUTCOME/MDD' 9. ' –q-score-range q.ranges.GWASsig_to_1 MDD_2019_pvalue_score' 10. ' –score MDD_2019_logORpVal 1 2 3 header no-mean-imputation' 11. " 12. '16384 MB RAM detected; reserving 8192 MB for main workspace.'

`--bfile` is to call in the PLINK binary files that we will need. This contains the 1000 Genomes samples

`--score` is to call in the effect size "Score File" from David Howard's 2019 MDD paper.

We also parse in a few arguments with this flag which are

`1 2 3` : where 1 is for the 1st column containing SNP ID, 2nd column is for the effective allele, and 3 is for the effect size estimate

`header` : is that the file has a header line

`no-mean-imputation`: we do not want our results for an individual's PRS to depend on the other individuals in the file

`--q-score-range` is to call in the p-value tranches/bins/groups "TRANCHE file" for grouping the p-values and p-values "p-value file" that we parsed in from David Howard's 2019 MDD paper

`--extract` is to compensate for genetic structure observed in humans called linkage disequilibrium

`--allow-no-sex` allows for plink to parse the phenotypes of data where sex is missing

`--out` is to print out the PRS computation

Where is our output?

[14]:
```
list.files(path="OUTCOME", pattern="MDD.P")
```

1. 'MDD.P05.profile' 2. 'MDD.P10.profile' 3. 'MDD.P20.profile' 4. 'MDD.P30.profile' 5. 'MDD.P40.profile' 6. 'MDD.P50.profile' 7. 'MDD.P75.profile' 8. 'MDD.Pe6.profile' 9. 'MDD.Psi.profile'

[15]:
```
list.files(path="OUTCOME", pattern="MDD.p")
```

1. 'MDD.pe2.profile' 2. 'MDD.pe3.profile' 3. 'MDD.pe4.profile'

2.4 Step 3: Analysis of MDD PRS to SSRI Response status

First, let's read the polygenic scores (`pe2` or p-value tranche of 0.001, i.e. anything lower than 0.001 is included) that we have previously computed and save it into an object named `dataset`

[16]:
```
dataset <- read.table("OUTCOME/MDD.pe2.profile",header=T)
```

[17]:
```
head(dataset)
```

FID	IID	PHENO	CNT	CNT2	SCORE
HG00096	HG00096	2	20824	9951	-0.000898552
HG00097	HG00097	2	20824	10033	-0.000864247
HG00099	HG00099	2	20824	9958	-0.000560535
HG00100	HG00100	2	20824	9826	-0.000698385
HG00101	HG00101	1	20824	10096	-0.000790333
HG00102	HG00102	2	20824	9977	-0.000402355

What's the range of the polygenic risk scores?

[18]:
```
summary(dataset$SCORE)
```

```
      Min.    1st Qu.     Median       Mean    3rd Qu.       Max.
-1.111e-03 -3.326e-04 -8.591e-05 -5.469e-05  2.383e-04  8.793e-04
```

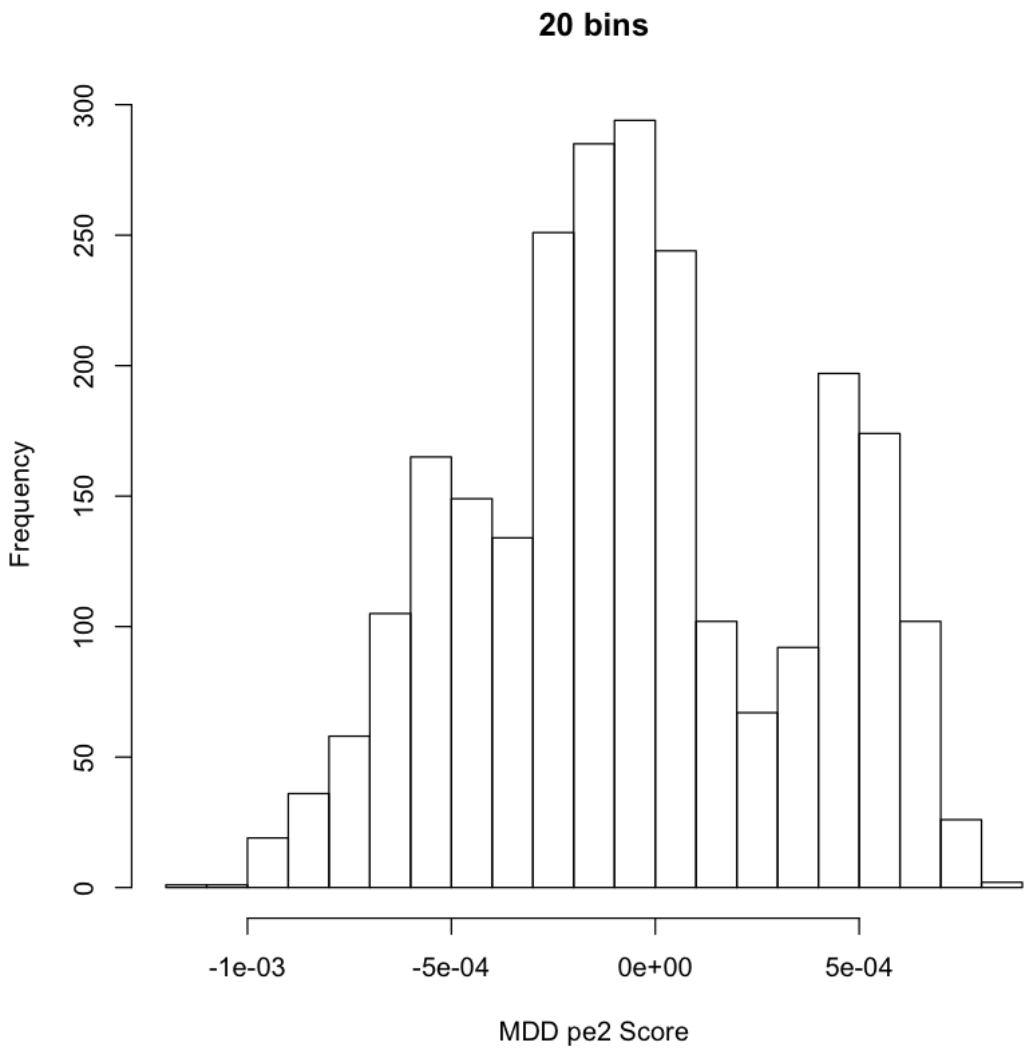
2.5 Step 3.1: Data exploration

Now, we will plot histograms of the PRS, and we will specify how many bins should be included (20 and 100).

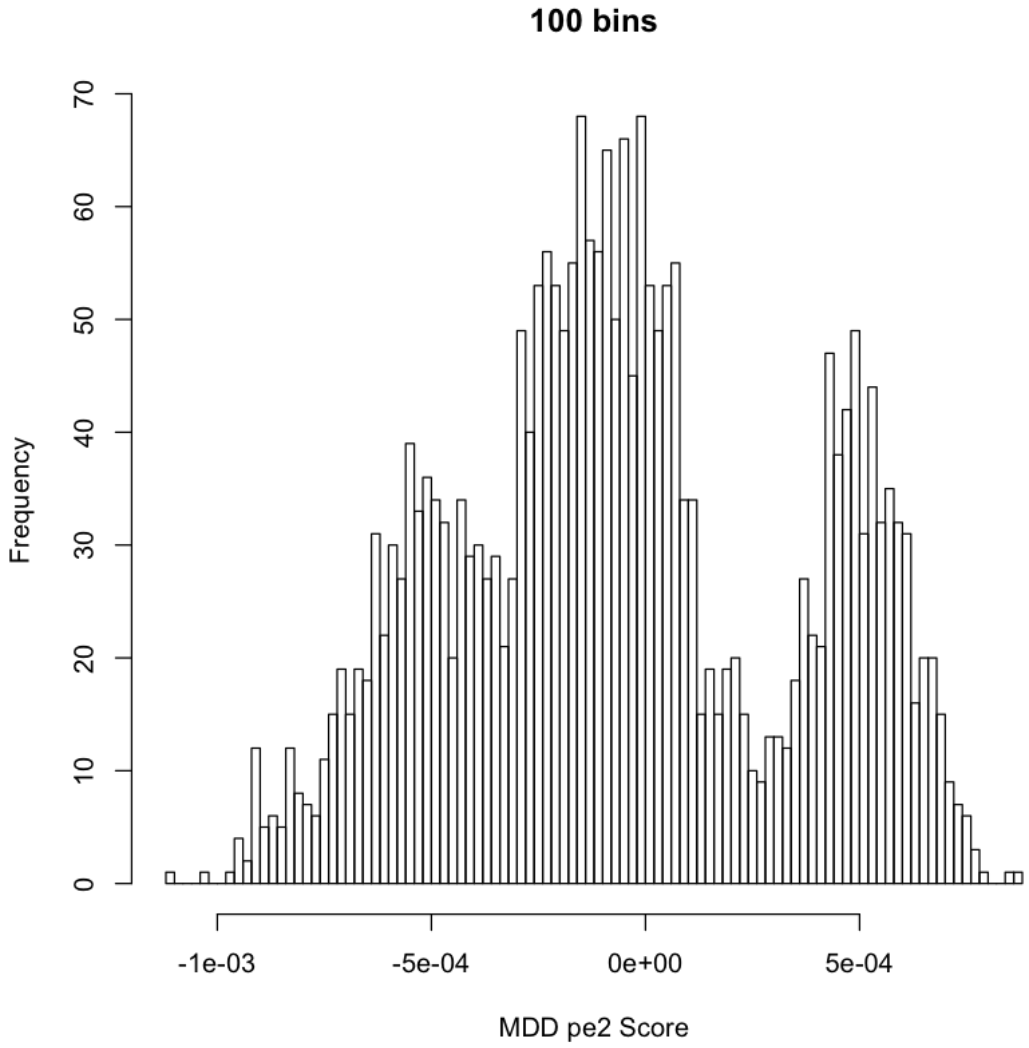
The additional commands below specify the title of the plot ('main=') and the label for the x-axis ('xlab='). It's always a good idea to plot your data throughout the analysis process. Histograms are one of my favourite methods.

using `R:base` to plot out how the histograms look like

[19]:
```
hist(dataset$SCORE, main="20 bins",xlab="MDD pe2 Score",breaks=20)
```



[20]:
```
hist(dataset$SCORE,main="100 bins",xlab="MDD pe2 Score",breaks=100)
```



Recall that we can use density plots to compare case/control status for polygenic risk load.

[21]:
```
## calculating the density function
noResponse <- density(dataset[dataset$PHENO==1,]$SCORE)
Response <- density(dataset[dataset$PHENO==2,]$SCORE)
```

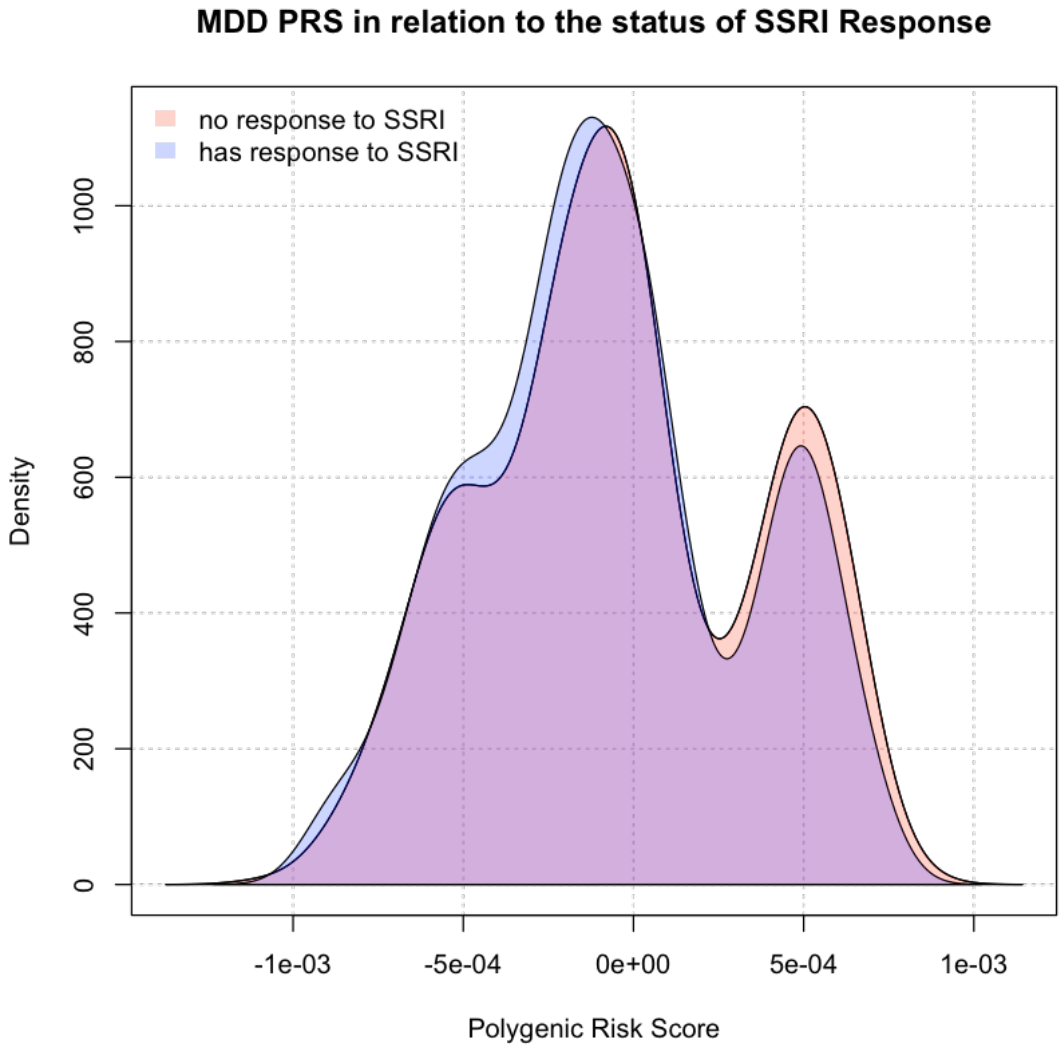
[22]:
```
## calculate the range for the plot
xlim <- range(noResponse$x, Response$x)
ylim <- range(0, noResponse$y, Response$y)
```

[23]:
```
#pick the colours
noResponseCol <- rgb(1,0,0,0.2)
ResponseCol <- rgb(0,0,1,0.2)
```

[24]:
```
## plot the no response plot and and set up most of the plot parameters
plot(noResponse, xlim = xlim, ylim = ylim, xlab = 'Polygenic Risk Score',
     main = 'MDD PRS in relation to the status of SSRI Response',
     panel.first = grid())

#put our density plots in
polygon(noResponse, density = -1, col = noResponseCol)
polygon(Response, density = -1, col = ResponseCol)

## add a legend in the corner
legend('topleft',c('no response to SSRI','has response to SSRI'),
       fill = c(noResponseCol, ResponseCol), bty = 'n',
       border = NA)
```



2.5.1 What do you think?

Is there a difference/shift in one of the groups? If yes, do you think it would imply a potential polygenic risk load?

2.5.2 Analysis of PRS? Yes/No?

Now that we see some slight difference, is there a statistical way to analyse this?

Why, yes! With something known as a regression analysis. A regression analysis allows us to test for whether a trait is associated with the PRS that we computed.

We can then report a p-value to see if the PRS is statistically significantly associated with the trait. In this case, "**is MDD PRS associated with SSRI response¿'**.

2.6 Step 3.2 Capturing ancestry differences in human

(Timing Check) Humans are different from each other! We need to control for this so that we can improve accuracy of modeling the trait that we plan on modeling.

To capture this, we use something known as principal components that were have already precomputed (or we did it in our workshop today).

[25]:
```
# Read the PC file into a file called 'pca'
pca <- read.table("OUTCOME/PCA_20_for_1000_Genomes.eigenvec")

# Provide the column names with this command.
names(pca) <- c("FID","IID",paste0("PC",1:20))

# Merge the polygenic score file (dataset) and the pca file (pca). Merging will␣
↪be based on the "FID" and "IID" columns.
dataset <- merge(dataset,pca,by=c("FID","IID"))
```

2.7 Step 3.3 PRS Analysis

Now that we have loaded the principal components, we can finally model our data (to find the association) for "**is MDD PRS associated with SSRI response¿'**.

We will be using something called a logistic regression analysis. Logistic because the trait that we are testing is a yes/no (binary/dichotomous). A regression analysis, in short would allow us to test whether there is an association of PRS to SSRI response.

We will run two regression analyses.

The first will include the polygenic score as the outcome, adjusting for population ancestry (using 10 PCs), and the variable that we are interested in which is PRS (SCORE):

[26]:
```
Fullmodel <- glm(data = dataset, PHENO-1 ~ SCORE + PC1 + PC2 + PC3 + PC4 + PC5 +
                    PC6 + PC7 + PC8 + PC9 + PC10, family = "binomial")
```

The second model only includes 10 PCs and *excludes* (reduced) the PRS (SCORE).

Why? We will need to use this for a later analysis in order to see what heritability (trait differences) are we estimating from the PRS

Later we will calculate how much phenotypic variance is explained by the full model (PRS + PCs) and how much phenotypic variance is explained by the reduced model (PCs only).

The difference in variance explained between the two models is the amount of variance explained by the polygenic score term. Other than association of a tested trait, this is the primary question that is usually asked in a polygenic scoring study.

[27]:
```
Reducedmodel <- glm(data = dataset, PHENO-1 ~ PC1 + PC2 + PC3 + PC4 + PC5 +
                      PC6 + PC7 + PC8 + PC9 + PC10, family = "binomial")
```

Let's examine the models

[28]:
```
summary(Fullmodel)
```

```
Call:
glm(formula = PHENO - 1 ~ SCORE + PC1 + PC2 + PC3 + PC4 + PC5 +
    PC6 + PC7 + PC8 + PC9 + PC10, family = "binomial", data = dataset)

Deviance Residuals:
   Min      1Q  Median      3Q     Max
-1.249  -1.179  -1.060   1.175   1.307

Coefficients:
              Estimate Std. Error z value Pr(>|z|)
(Intercept)   -0.04311    0.04307  -1.001    0.317
SCORE       -111.72604  289.59694  -0.386    0.700
PC1           -3.30588    4.91131  -0.673    0.501
PC2           -0.68915    3.43034  -0.201    0.841
PC3            0.48982    2.22353   0.220    0.826
PC4           -0.06881    2.19792  -0.031    0.975
PC5           -0.38738    2.00784  -0.193    0.847
PC6           -0.89865    2.00377  -0.448    0.654
PC7           -1.61608    2.00859  -0.805    0.421
PC8            1.27905    2.01003   0.636    0.525
PC9            0.60880    2.00185   0.304    0.761
PC10          -1.60740    2.00765  -0.801    0.423

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 3470.4  on 2503  degrees of freedom
Residual deviance: 3461.6  on 2492  degrees of freedom
AIC: 3485.6

Number of Fisher Scoring iterations: 3
```

[29]:
```
summary(Reducedmodel)
```

```
Call:
glm(formula = PHENO - 1 ~ PC1 + PC2 + PC3 + PC4 + PC5 + PC6 +
    PC7 + PC8 + PC9 + PC10, family = "binomial", data = dataset)

Deviance Residuals:
   Min      1Q  Median      3Q     Max
-1.236  -1.179  -1.063   1.175   1.301

Coefficients:
            Estimate Std. Error z value Pr(>|z|)
(Intercept) -0.03700    0.04004  -0.924   0.3555
PC1         -5.03497    2.00970  -2.505   0.0122 *
PC2          0.38581    2.00101   0.193   0.8471
PC3          0.86421    2.00060   0.432   0.6658
PC4          0.28212    2.00081   0.141   0.8879
PC5         -0.45073    2.00105  -0.225   0.8218
PC6         -0.93826    2.00109  -0.469   0.6392
PC7         -1.63442    2.00798  -0.814   0.4157
PC8          1.26208    2.00949   0.628   0.5300
PC9          0.60320    2.00171   0.301   0.7632
PC10        -1.66414    2.00219  -0.831   0.4059
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 3470.4  on 2503  degrees of freedom
Residual deviance: 3461.8  on 2493  degrees of freedom
AIC: 3483.8

Number of Fisher Scoring iterations: 3
```

2.8 Step 3.4 Phenotype/ Trait Variance that is explained by PRS

Let's now calculate the phenotype / trait variance (that's sometimes also known as heritability) explained by the polygenic scoring 'SCORE' term.

We will be using a function from a package that we initially loaded. The package is called `fmsb` and the function that we will be using is called `NagelkerkeR2`.

Nagelkerke's R2 is a metric that we use to explain how much of a trait tested is measured by PRS. This ranges from 0-1.

We would usually hope and pray and wish it's as close to 1 but in reality, as we covered at the top of this workshop – barely 20% is ever explained!

This command now calculates Nagelkerke's R2. The subsequent command extracts just the R2 value.

[30]:
```
Fullmodel.rsquare <- NagelkerkeR2(Fullmodel)[[2]]
Fullmodel.rsquare
```

0.00467737481470882

Similarly, let's compute Nagelkerke's R2 for the reduced model

[31]:
```
Reducedmodel.rsquare <- NagelkerkeR2(Reducedmodel)[[2]]
Reducedmodel.rsquare
```

0.00459837968804138

Each of the values above explain variances in each model. Now, the rationale behind using reduced vs full model comes in.

We will take the difference between the two models to see how much is the variance being explained by MDD PRS to SSRI Response.

Let's now compute the R2 difference between the models.

[32]:
```
diff.rsquare <- Fullmodel.rsquare -  Reducedmodel.rsquare
```

2.9 Step 3.5 Results (FINALLY)

Let's now pull all of the results into one file so that we understand what our data is telling us.

First, we will obtain the full model's (including PRS) effect size and p-value for the polygenic score term. Note, again, that this is not significant.

[33]:
```
Estimate <- coef(summary(Fullmodel))[2,c(1,4)]
```

Next, we will place these values into a `results` file that contains the difference in R2 `diff.rsquare`

In the subsequent `names(results)` line, we can name column headers manually in order to improve clarity.

[34]:
```
results <- as.data.frame(cbind(Estimate[1],Estimate[2],diff.rsquare))
names(results) <- c("OR","p-value","NagelkerkeR2")
```

So, what is the summary of our results?

drum roll please

[35]:
```
results
```

	OR	p-value	NagelkerkeR2
Estimate	-111.726	0.699646	7.899513e-05

3 Breakout session

If we have the time, we will try out some practical assignments that we do in genetics.

As we eluded to earlier, the variance explained by PRS towards a trait is one of our primary goals. Based on the model that you have ran with the `pe2` tranche, we noticed an R2 of 7.899513 x 10-5.

Do you think that this will *increase* when we loosen up the threshold i.e. use `pe05`, `pe10`?

This would allow **more SNPs** to be used as reference from the reference data into our test data.

Do you think that this will *decrease* if we make this threshold more stringent i.e. use `pe4`, `pe6`?

This would allow **less SNPs** to be used as reference from the reference data into our test data.

Let's test this out!

3.1 Breakout room 1: Testing the INCREASE in p-value tranche threshold

Use `pe10`

File to use: `OUTCOME/MDD.P10.profile`

3.2 Breakout room 2: Testing the DECREASE in p-value tranche threshold

Use `pe4`

File to use: `OUTCOME/MDD.pe4.profile`

3.3 What do you have to do?

1) Introduce yourselves!
2) Identify a note-taker (and a back up, just in case). This person will also share their screen with the group for code reviewing.
3) Identify a reporter who will share your group's responses with the larger group.

Kumar will pop in and out of your rooms to check in; please use the "Ask for Help" button to bring Kumar into your group as and when needed

3.3.1 Steps-by-step guide

1) Copy and paste the analysis steps for **Step 3** (Steps 3.1 through 3.5) into separate cells below this cell. ***OR*** you can just edit the above cells. It's really up to you!
2) Choose the appropriate files to run your analysis. All you would have to do is substitute with the appropriate p-value tranche threshold that you were assigned. The command that you would edit is `dataset <- read.table("OUTCOME/MDD.pe2.profile",header=T)`
3) Within your group, discuss the

3.1) effect size and p-value contributed by the MDD PRS to SSRI response

3.2) Is the effect size the same?

3.3) More importantly, what is the Nagelkerke's R2 and how does it compare to `pe2`? What do you think this means?

3.4) Can genetics be any more complex than this? *groan*

We will broadcast a 2-minute warning when it's time to start wrapping up.

4) Present your group's work by sharing the screen of your reporter. *Each group will have 3-5 minutes to share their discussion points.*

[ ]: