DEVELOPMENT OF A BIOINFORMATIC APPROACH TO IDENTIFY CANDIDATE PATHOGENIC VARIANTS IN CANINE PHARMACOGENOMIC GENES

Master's Thesis

Sruthi M Hundi

Faculty of Medicine and Life Sciences

University of Helsinki

February 2017

Acknowledgment

This work was conducted at the Canine Genetics research group led by Professor Hannes T Lohi, from the Department of Veterinary Science at the University of Helsinki. I would like to thank Professor Hannes Lohi for giving me the opportunity to conduct the research at this lab and his continuous supervision.

My sincere thanks to Marjo K Hytönen, as this study would have been more difficult if not for her motivation and advice. I am thankful to Meharji Arumilli, for his valuable inputs on NGS data analysis. I also extend my gratitude to all lab members of Hannes Lohi for a very friendly work environment.

Finally I wish to thank my entire family, especially my parents Chandra Mouli and Usha Mouli, and my husband Venkatram Yellapragada for being the key source of my strength.

Tampere, February 2017
Sruthi M Hundi

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ABC	ATP Binding Cassette
ADME	Absorption Distribution Metabolism Excretion
BAM	Binary Alignment Map
BWT	Burrows Wheelers Transform
CPIC	Clinical Pharmacogenetics Implementation Consortium
CYP	Cytochrome P-450
IGV	Integrative Genome Viewer
ITPC	International Tamoxifen Pharmacogenomics Consortium
IWPC	International Wafarin Pharmacogenomics Consortium
MDR	Multi Drug Resistance
MSA	Multiple Sequence Alignment
MUSCLE	Multiple Sequence Alignment by Log Expectation
NGS	Next Generation Sequencing
OMIA	Online Mendelian Inheritance in Animals
OMIM	Online Mendelian Inheritance in Man
PGx	Pharmacogenomic
PharmGKB	The Pharmacogenomic Knowledge Base
SLC	Solute Like Carrier
VCF	Variant Call Format
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing

Master's Thesis

Place: Faculty of Medicine and Faculty Sciences,
University of Tampere

Author: Sruthi M Hundi

Title: Development of a Bioinformatics Approach to Identify Candidate Pathogenic Variants in Canine Pharmacogenomic Genes

Pages: 55

Supervisor: Professor Hannes T Lohi, Department of veterinary medicine,
University of Helsinki.

Reviewers: Professor Matti Nykter, Faculty of Medicine and Life sciences,
University of Tampere.
Professor Hannes T Lohi, Department of veterinary medicine,
University of Helsinki.

Date: 22nd February 2017

Abstract

Genetic variations in pharmacogenomic genes result in diverse response of individuals to different drugs. Understanding the functional implications of these variations has gathered a significant research interest over the past decades providing a prime example for personalized medicine. Personalized medicine is a rapidly evolving field of pharmacogenetics that involves individual design of drug composition and dosage based on the genetic profile. The annotation of the genomes of domestic animals such as dog followed by an increasing amount of available whole genome sequencing data opens new opportunities for pharmacogenomics (PGx) in animal models. Despite some highlighted examples of canine PGx, e.g. MDR1 susceptibility, canine PGx is still poorly characterized.

The major aim of this thesis was to utilize the growing number of WGS (Whole Genome Sequence) data for PGx profiling by developing a bioinformatic analysis pipeline that can identify potential candidate pathogenic variants. Canine orthologs for 540 Human PGx genes were retrieved resulting in 495 canine PGx genes. Ensembl's phylogenetic trees were used to identify the orthologs. The pipeline analysis was piloted in 24 dogs in Border collie. A pipeline was developed to analyze the WGS of these dogs and to identify the pathogenic variants. The analysis altogether revealed 2964 variants in the coding regions of these 495 Canine pharmacogenomic genes. Out of these, 56 variants (1.8%) were predicted to be pathogenic and could be prioritized for further validation to determine their prevalence and functional significance. A pharmacogenomic annotation of these genes was also established, using available human data as a reference model. This annotation categorizes them based on their ortholog relationship, role in drug processing and importance in pharmacogenomics.

1. Introduction

It has been known for centuries that not everybody reacts to a medicine in a uniform fashion. In the 1500s, Philippus Paracelsus said 'Medicine is not merely a science but is an art'. Sir William Osler stated in 1892 that 'If it were not for the great variability among individuals, medicine might as well be a science and not an art'. Several genes, generalized as *pharmacogenes* are involved in the life cycle of a drug in a body. Pharmacogenomics (hereafter referred as PGx in the document) is the study of how genomic variation in these genes influences the pattern a drug is processed in a body. These pharmacogenes play an important role in processing of the drug that includes Absorption, Distribution, Metabolism and Excretion (ADME) (Johnson, 2003). Genetic variability has a significant influence on how a drug mechanism works in a body and mutations in these genes can thus be a major causative factor for distinctive response to a drug (Shin, Kayser, & Langaee, 2009) as shown in Figure 1. For example, a mutation in a drug receptor-encoding gene could result in an altered protein, affecting the process of absorption of the drug and eventually the effect of the drug.

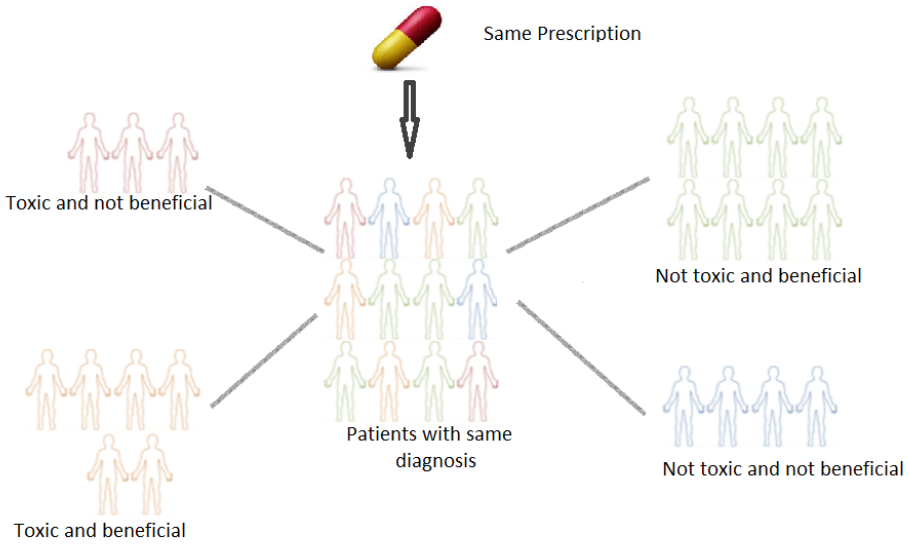


Figure 1 *An illustration on, different people respond differently to the same therapy*

However, a mutation in the pharmacogenes can be pathogenic only when administered with drugs, although the individual may not display any disease specific phenotype in absence of the drug. The ability to tailor the prescription, so that there is an increase in the probability of beneficial outcomes and a decrease in the probability of negative effects, has led to increasing

interest towards 'personalized medicine' (Chan & Ginsburg, 2011). This field also focuses on preventive and prediction medicine, at a reduced cost, rather than responsive medicine.

PGx intends to customize the medical treatment as per their individual genome and uses bioinformatics approach to study their genomic profile. The basic idea of personalized medicine revolves around, the concept of, *connecting information from traditional medicine with personal genomics*. While traditional medicine defines the relationship between phenotype (pathogenic state) and the medical treatment, personal genomics provides information on phenotype and genotype correlation. As can be seen in Figure 2, pharmacogenomics connects genotype information from personal genomics, medicinal information from translation medicine and draws a meaningful relationship between them.

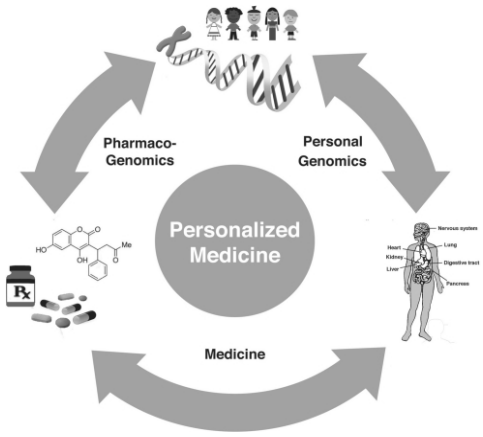


Figure 2. *Personalized medicine connecting genotype, phenotype and medicine (Fernald, Capriotti, Daneshjou, Karczewski, & Altman, 2011a).*

Bioinformatics plays an essential role in various stages in the process of making personalized medicine a reality (Rodriguez-Antona, 2015). The exponential increase in the use of sequencing technologies and reduction in its cost has enabled the use of next generation sequencing (NGS) in many genetic studies. As a result there is an increased availability of personal genomic data. A bioinformatic approach to resourcefully use the available NGS data, to yield clinically relevant information is a multistep process as shown below in Figure. 3 (Fernald, Capriotti, Daneshjou, Karczewski, & Altman, 2011b).

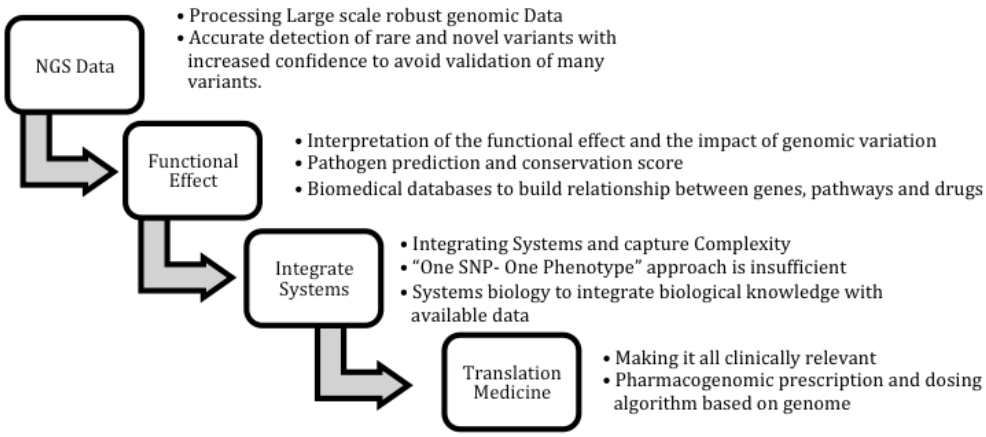


Figure 3. *A schematic overview of pipeline to achieve translation-medicine from NGS data.*

It would thus be of great necessity to have a bioinformatic pipeline that can serve this need. This project is a start to suffice the first two steps of the pipeline depicted in Figure. 3 and study the variance and mutations in pharmacogenes using the canine genome as a model of study. The process is documented in the next chapters of this thesis. In chapter 2, Literature review, the essential background information to understand the thesis and key finding in the same line of research is reviewed. In the chapter 3, Aims and Objectives, the overall aim and specific objectives of the study are listed. In chapter 4, Methods and materials, the pipeline developed and algorithms adapted in the pipeline are described. In chapter 5, Results, the finding obtained by applying the pipeline to a set of data is recorded section. In chapter 6, Discussion, along with discussing the findings, the efficiency and the limitations of the pipeline is also discussed. The results are also compared with the present on going research in this chapter. Finally in chapter 7, labeled as Conclusion, the possible options of optimizing the tool to address the limitations and the future prospects of research are discussed.

2. Literature Review

A drug that is administered needs to be absorbed at proper levels, distributed to targeted tissues, metabolized and then excreted from the body, as is the process of ADME. A mutation in a gene that encodes the drug processing protein can interfere the process of drug metabolism or sometimes may also cause toxic effects. Information on such mutations is the key to identify or design biomarkers that can qualitatively and quantitatively predict the drug response (Frank & Hargreaves, 2003). There are several PGx databases that have been striving to identify, store and deliver such important information of the pharmacogenomic genes, their variation and relationships such as gene-drug, gene-pathway etc. Some of the very well known databases are PharmGKB (Thorn, Klein, & Altman, 2005, 2010) and pharmaADME (http://www.pharmaadme.org).

PharmGKB is a pharmacogenomic database that started as an effort to store post-genomic data in 2000 (Thorn et al., 2010). With the advent of new technologies, the flow of data has exploded, and since then, PharmGKB refocused to employ knowledge and capture complex relationship between genes, drugs, pathways and variations. All the gathered information is organized, stored and labeled based on different criteria such as pharmacokinetics, pharmacodynamics, cellular component, molecular function, clinical significance (disease/phenotype), importance in drug processing and availability of genotype data. The data can be accessed with respect to these labels or the gene. About 400 pharmacogenes have been listed as a part of various collaborative projects such as Clinical Pharmacogenetics Implementation Consortium (CPIC) (Relling, 2015), International Tamoxifen Pharmacogenomics Consortium (ITPC) and International Warfarin Pharmacogenomics Consortium (IWPC) (International Warfarin Pharmacogenetics Consortium et al., 2009). Several well-known examples include Cytochrome P450 (CYP) drug-metabolizing family genes in the liver, as well as genes in the ATP-binding Cassette (ABC) transporters and Solute Carrier (SLC) transporter families.

PharmaADME is another well-known pharmacogenomic consortium that makes effort to develop standardized evidence based drug metabolizing genetic biomarkers. The biomarkers are used in the process of drug development to predict genetic and pharmacokinetic (the rate at which the drug is processed) variability in an individual body. PharmaADME has categorized genes as core, extended and related genes based on its importance and relatedness

with drug processing. There are also databases specific to some very important PGx genes and alleles such as CYP-allele database (S. C. Sim & Ingelman-Sundberg, 2013), NAT-allele database (E. Sim, Fakis, Laurieri, & Boukouvala, 2012) and TP search database (Ozawa et al., 2004). Combining the information from these databases with genomics would leadoff genotype to phenotype research in contrast to the traditional phenotype to genotype mode of approach. This mode of research was tried out successfully to predict drug sensitivity phenotypes by using genotype information of CYP2D6 from CPIC (Gaedigk, Sangkuhl, Whirl-Carrillo, Klein, & Leeder, 2016).

Most of these databases are aimed at pharmacogenomics of human genome. However, the availability of genome assembly and advances in sequence data annotations for domestic animals is making it possible to make significant finding in veterinary pharmacogenomics. Court et al suggest that using the advancing bioinformatic technologies and comparative genomics, it is also possible to relate knowledge from the above databases to contribute to comparative medicine (Mosher & Court, 2010).

2.1 **Dog as a model for Pharmacogenomics**

A dog (*Canis lupus familiaris)* characterizes as an interesting model for both genetic and pharmacogenetic study. The whole genome of human and dog are about 95 percent similar. Most of the genes in humans have an orthologous or a predicted gene in the dog. Both human and dog follow a similar disease inheritance pattern and the mutated gene for a disease is often the same. Several findings in dogs have helped to identify genetic conditions in humans such as in cancer (Ranieri et al., 2013) and neurological disease (Seppälä et al., 2011). The genetic distance in different breeds of dogs is much higher (3-4 x) than the genetic diversity in different human populations. Breed-specific mutations in PGx genes are very important as these mutations could have toxic effects against the administered drugs (Fleischer, Sharkey, Mealey, Ostrander, & Martinez, 2008). Along with genomic features, environmental factors also play a huge role in personalized medicine (Ginsburg & Willard, 2009). Dogs share the same environment with humans and hence are exposed to very similar environmental factors. Thus, dogs can be considered as an important model for pharmacogenetic study.

Although the pharmacogenomics research in dogs is still not as advanced, as in human, it is one of the most well studied animals with clinically relevant pharmacogenetic discoveries. Many variants that could be pharmacogenomic related and pathogenic have been identified (Katrina L Mealey, 2006). The key transporter and metabolizer genes well studied in canine include, the ABC transporter and CYP family genes. Some of the prominent discoveries in canine PGx include the MDR1 (Multi Drug Resistance) delta mutation(Katrina L Mealey, 2013), Cytochrome P-450 variants (Court, 2013) and the TPMT variation (Salavaggione & Kidd, 2002).

The P-glycoprotein (P-gp) produced by the MDR1 gene is a key transporter protein (Zhou, Gottesman, & Pastan, 1999). The well-known four base pair deletion in MDR1 shifts the ORF leading to generation of premature stop codon (K L Mealey, Bentjen, Gay, & Cantor, 2001). This produces a malformed or mutated P-gp protein that is about 10% in length of the normal P-gp protein. The affected dogs are said to exhibit the "multidrug sensitivity" phenotype. These dogs exhibit high difference in distribution and excretion of the drug when compared to the normal ones. Studies have shown that the normal dogs could show neurotoxicity at high doses (>2 mg/kg), while heterozygous and homozygous mutants showed neurotoxicity at lower doses, approximating at 300 micro g/kg dose and 129 micro g/kg respectively (K L Mealey et al., 2001). In another study a Collie with hetero MDR1 mutation affected with lymphoma when treated with doxorubicin, a P-gp substrate, exhibited gastro intestinal toxicity. It was inferred in this study that improper excretion of the drug could have been the causative factor of the toxicity (K. L. Mealey et al., 2008)

CYP is an essential drug metabolizing and excreting gene. The CYP genes CYPB11 is said to be highly variable among different dog breeds (Court, 2013). However, the clinical impact of this is not yet known. There are also other possible pathogenic variants identified in dogs but with unknown clinical significance such as CYP2C41 gene deletion and amino acid variants in CYP2D15, CYP2E1 and CYP3A12. A genetic variation in the CYP2D15 gene, which processes the drug Celecoxib, includes deletion of an exon 3. This deletion leads to unidentified metabolism of Celecoxib. The other noted polymorphism in this family is the CYP1A2 premature stop polymorphism. The premature stop codon causes loss of enzyme activity of the gene. In the study by Court et. al, the affected dogs seemed to contain high level of the respective substrate drug, when compared to the normal dogs, depicting low or no enzyme activity of the impaired gene (Court, 2013).

TPMT is a Phase II metabolizing enzyme that has a pharmacogenomic-identified variant. This variant, causes decreased enzymatic activity of its substrate azathioprine leading to high susceptibility to azathioprine-induced suppression of bone marrow (Haller et al., 2012).

2.2 Role of Next Generation Sequencing in Canine PGx study

To make reliable PGx predictions based on individual genomes and gathered genetic evidence, the first essential requirement as depicted in Figure 2, is to accumulate genomic data. DNA sequencing is the process of resolving the DNA sequence from a sample. The history of DNA sequencing hails back to 1965 when Holley sequenced yeast tRNA. Sanger sequencing was a major breakthrough for DNA sequencing as it laid foundations to the process of First Generation Sequencing or Automated Sanger sequencing. Ever since many improvements have been made at a faster pace in the process of sequencing leading to the advent of NGS methods.

Table 1. History of DNA Sequencing Adapted from (Messing & Llaca, 1998)

Efficiency(bp/person/year)	**Year**	**Breakthrough in Sequencing**
	1870	Miescher: Discovers DNA
	1953	Watson &Crick: Double Helix structure of DNA
1	1965	Holley: Sequences Yeast tRNA
1500	1977	Sanger Sequencing
50,000	1990	Cycle (Fluroscent) Sequencing
50,000,000 - 100,000,000,000	2002-2008	Next Generation Sequencing

With the exponential decrease in the cost of NGS analysis, the accumulation of genomic sequence data across species is less of a challenge today (Figure 4). For the past 15 years, the cost of sequencing per genome has drastically reduced from 100 million dollars to about 1 K dollars.

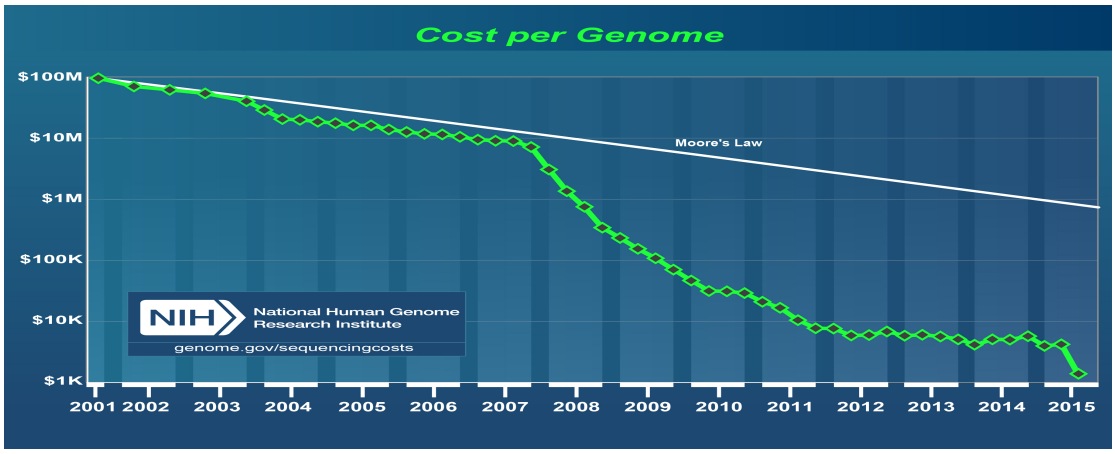


Figure 4. *Data from NHGRI describing the reduction in cost for sequencing per genome ("The Cost of Sequencing a Human Genome," n.d.)*

The first Whole Genome Sequence (WGS) was accomplished in the year 1995 by Fleischmann et.al, when they published the complete sequence of Heamophilus influenza- a common bacteria present in the respiratory tract of humans. The application of WGS saw a rapid rise in the early 2000s when human and mouse and many other mammals were sequenced (Waterston et al., 2002).

The first version whole Canine genome was sequenced in Standard Poodle in 2003, and was followed by a higher quality sequence in Boxer in 2005. This was referred as the first canine genome assembly, CanFam1.0. The assembly was updated to CanFam2 in 2005 (Lindblad-Toh et al., 2005). The latest updated version of canine assembly is CanFam3.1 that was published in 2012. Besides WGS, methods such as whole exome sequencing (WES) and targeted resequencing that includes sequencing of only the exome of a genome and a specific region of genome, respectively, are also widely used in dogs nowadays (Ahonen et al 2013) .

Once the genome is sequenced either through WGS, WES or targeted resequencing, the produced data can be used to perform downstream NGS data analysis. However, there are many challenges that need to be overcome to obtain reliable and reproducible data (Shendure & Ji, 2008). Improper quality of the NGS data is a significant challenge that needs to be addressed. Many issues such as poor quality of the sequencing technique leading to increased error in base pair calling, low coverage and low quality reads are a few to mention (Yu & Sun, 2013). After sequencing data quality control, the identification of novel variants should be a performed with increased confidence, to avoid false positives and detect the variants,

which are otherwise falsely tagged as negatives(Nielsen, Paul, Albrechtsen, & Song, 2011). For this use of dbSNP and in-house variants are essential.

The WGS data of a sample includes 3-4 millions variants. However, not all of these variants are pathogenic. Particular bioinformatic and functional approaches are required to predict and confirm the pathogenicity of the variants. Most of the pathogenic genomic variants alter the amino acid sequence and subsequent protein structure affecting its proper function. A study suggests that protein molecules are quite robust and tolerate small changes in the amino acid sequence (M Pajunen et al.). However, if an amino acid or multiple amino-acid changes happen to alter a property of the protein such as structure (Feyfant E et.al) of protein or catalytic activity of protein (Yusuke Takahashi et.al), then it could be pathogenic. Certain mutations could happen to change the function of a protein (M Oren et al), i.e. if a protein has a ligand binding capacity, loss of function would lead to improper binding to ligand, while gain of binding would lead to unnecessary binding to ligand. Hence, understanding the impact of the mutation on the protein structure and function is essential to be able to evaluate the pathogenicity of the mutation. For the prediction of the possible impact of the mutation, evolutionary analysis is an approach, based on the assumption that a change in a conserved position does not allow alterations without a compromise on function. However, the bioinformatic methods can only do the predictions and the true pathophysiology of the mutation has to be confirmed experimentally (Fernald et al., 2011b) .

3. Aims of the study

Our hypothesis is that extensive genomic variation exist in canine PGx genes at individual and breed level and that the most likely pathogenic variants lie within the conserved functional regions of the open reading frames (ORFs) altering the protein structure and functions. To test this hypothesis in a pilot study cohort of 24 Border Collies, we included the following specific aims:

- Retrieve a list of known human PGx genes and their annotations such as pharmacogenomic functions and processed drugs utilizing available public databases such as PharmgKb and PharmaADME.
- Identify canine PGx orthologs.
- Build a bioinformatic pipeline to analyze the WGS data of 24 Border Collies to identify genomic variants and their predicted implications in canine PGx genes.
- Analyze the frequency and significance of the variants in the breed.

4. Materials and Methods

The process and flow of the project, starting from detecting the pharmacogenetic genes, to discovering the possibly pathogenic variants and genes, are described in detail in this section.

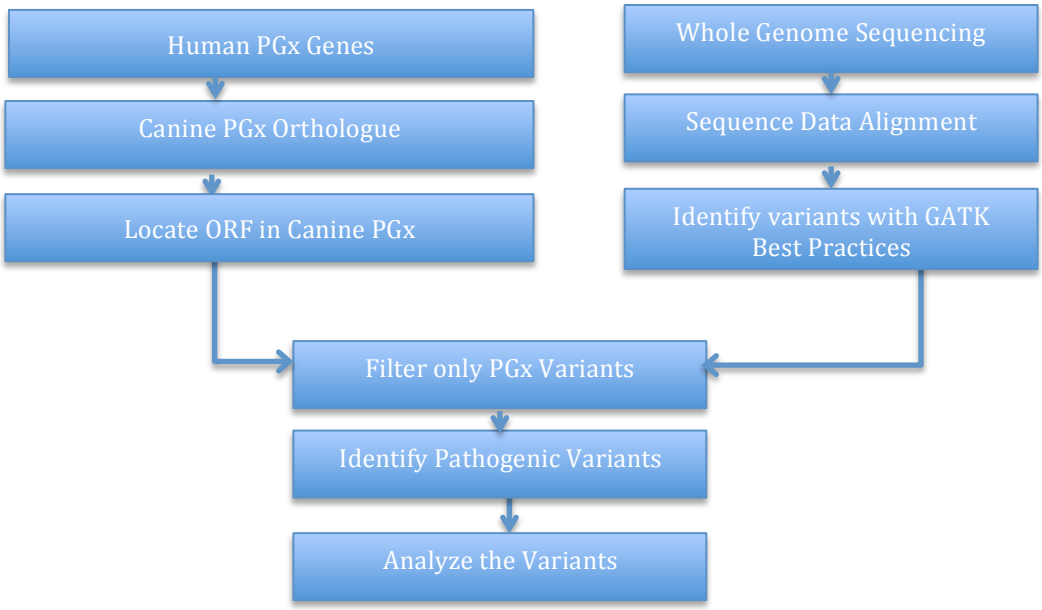


Figure 5. *The schematic overview of the proposed pipeline, which involves two different sections; One section involves identification of Orthologue for the Canine PGx genes and the other section involves WGS data analysis to identify pathogenic variants for the PGx genes from section 1.*

4.1 Human – Canine Pharmacogenetic Genes

PharmGKB and pharmaADME are selected for this study as these two databases contain information about pharmacogenetics on a generic level, unlike specific gene families databases such as CYP, NAT, etc., (S. C. Sim, Altman, & Ingelman-Sundberg, 2011) or drug transporters and modifier specific databases. From PharmGKB, genes from sections *PharmgKb- Drugs and genes* and *CPIC* (Clinical Pharmacogenetics Implementation Consortium) genes were included. Genes that are considered to play a key role in pharmacogenomics by prof. Mikko Niemi at the Department of Pharmacogenetics, University of Helsinki, were also included in the study. The genes from all the four sources were retrieved and consolidated to create a list of Pharmacogenetic genes.

4.2 **Canine orthologues**

The canine orthologues were retrieved using Ensembl Compara gene trees (Vilella et al., 2009). Ensembl Compara is a computation pipeline that was built to produce phylogenetic trees for many genomes, especially the vertebrates, evolved during the process of evolution. The general idea of evolution is that all forms of life shares a common ancestor and this primary theory is defined as 'descent with modification'. Sweeping changes in the genome has led to the process of speciation throughout evolution. Even after speciation, many genes or proteins sharing the same functionality remain highly similar. A phylogenic tree is more like a graph of the evolutionary journey of a gene from root to different species (Doolittle, 1999). Species that are genetically close lie close to each other or belong to the same cluster in a phylogenetic tree. To perform comparative analysis on an interested gene, the first essential step is not only to identify the homologous gene, but also to identify the type of the relationship of the gene between the species, i.e. whether it is in an ortholog or a paralog. This information is readily available from a phylogenetic tree or a gene tree.

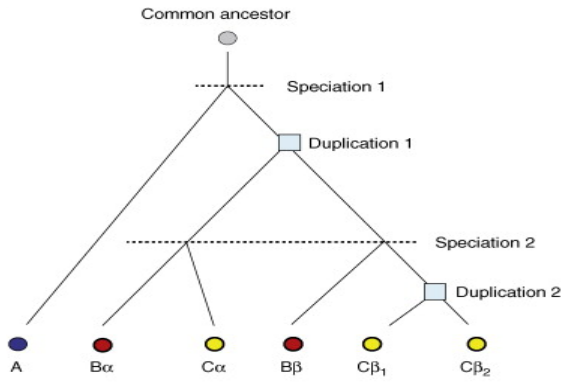


Figure 6. *Example of phylogenetic tree adapted from (Studer & Robinson-Rechavi, 2009)*

Table 2. Gene relationship in gene from different species

Gene	Pair	Relation-Ship	Type
A	All others	Orthologue	One-to-many
α	Bα and Cα	Orthologue	One-to-One
β	Bβ, $C\beta_1$ and $C\beta_2$	Orthologue	One-to-many
B	$C\beta_1$ and $C\beta_2$	Paralogue	Within-species

The Ensembl Compara gene trees were created using a series of computational steps that starts with protein sequences of all species and ends with creation of gene tress. The protein sequences were obtained, from all the species involved in the study, by retrieving the longest translation available in the Ensembl database. The protein sequence from each species was analyzed with pBLAST against protein sequences of all other species protein databases. Based on the BLAST Score Ratios (BSR) (Ratio of blast score between two species), proteins of different species were connected to form a graph. From the graph the clusters were

identified using linkage cluster methods. All the proteins in a single cluster belonged to a single gene family. The protein sequences of all these were performed Multiple Sequence Alignment (MSA) using MUSCLE. The protein sequence obtained from MSA was back translated to DNA sequence, and was given as an input to the program, TreeBeST, that generated the required phylogenetic tree.

Ensembl Perl API consists of four connected databases known as Core, Compara, Variation and Regulation. Each database uses different classes known as adaptors to retrieve the required information. The homology adaptor from Compara was used to retrieve the ortholog information between human and dog. Along with the orthologue gene, information such as eValue and dn/ds ratio was also retrieved.

4.3 **Identifying ORF in Canine PGx Genes**

For the canine orthologs retrieved from Ensembl Compara, the genomic co-ordinates for only the protein coding regions needed to be obtained. As per the central dogma of molecular biology, the DNA is transcribed into mRNA (Introns + Exons + UTR), mRNA in-turn into mature mRNA (Exons +UTR) after the splicing event. Maximum portion of the mature mRNA is comprised of the coding sequences that are translated to proteins. This region of mRNA that is translated to protein comprises the open reading frame (ORF, Figure 7).

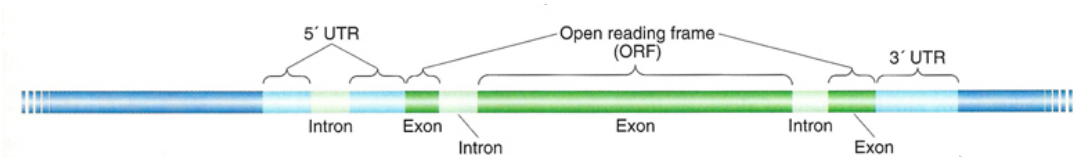


Figure 7. *Up stream , downstream, intron , exon and ORF explained*

By using the information of ortholog gene retrieved (from the homology adaptor), gene specific information was retrieved using the gene adaptor from the Ensembl core database. The information included details of the gene such as genomic location, co-ordinates of exons and coding regions within the gene. Using this information a bed file was created with only the open reading frames (of the longest transcript) of each ortholog genes.

4.4 Whole Genome Sequencing

DNA samples were collected from twenty-four Border Collies. DNA was isolated from the EDTA-blood using semi-automated Chemagen robot and purified. DNA prepared and sequenced using Illumina HiSeq2500 methodology as described by Hytönen et al 2016, PlosGenet.

4.4.1 Illumina Sequencing

Illumina is a widely adopted sequencing platform. The complete process of Illumina sequencing can be divided into four stages: sample preparation, cluster generation, sequencing and data analysis. Libraries are constructed that contains adaptors to be attached to the DNA during sequencing. Paired end approach was used, as it reads from both ends of the DNA. In this approach both ends of DNA are ligated with adaptors to make sure that both ends are read during sequencing. The adapters on either end of the DNA fragment are complementary to each other (Figure 8).

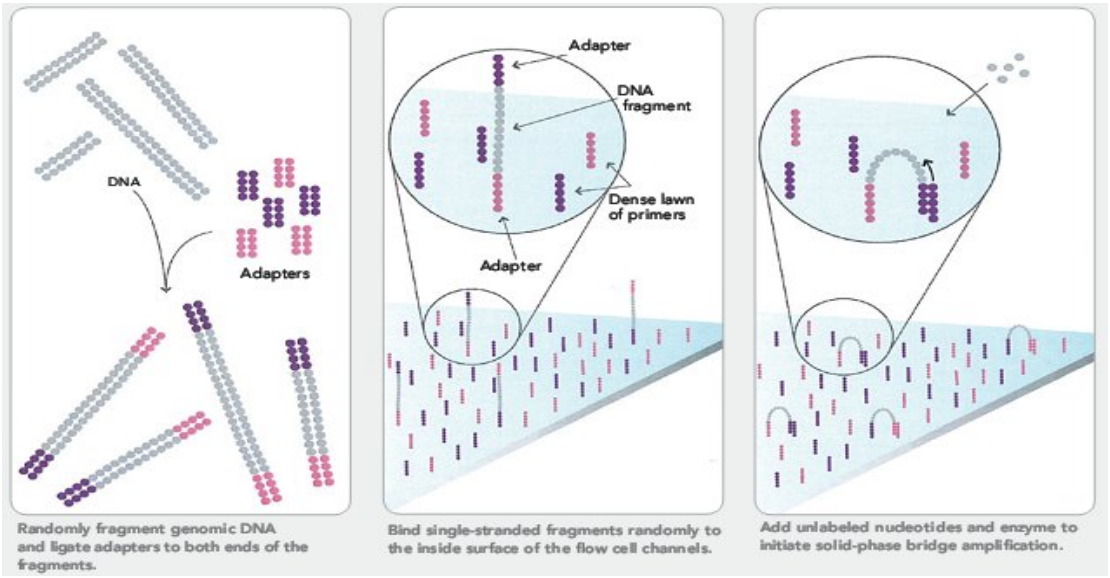


Figure 8. *Ligation of adaptors to DNA and binding to the flow cell. Retrieved from illumina.com*

These ligated DNA fragments undergo amplification and are then bind to the flow cell. The flow cell is a slide that has multiple lanes. On these lanes a number of oligonucleotides are attached to the floor of the flow cell. There are two types of oligo nucleotides and they are complementary to each other and to the adaptors ligated to the DNA fragments. The DNA is hybridized to the flow-cell with the complimentary oligos and adaptors. A polymerase creates

a complementary strand to the hybridized DNA creating a double stranded DNA. From this double stranded DNA, the original strand is detached by washing it away. The free end of the newly created complementary strand also binds to the flow cell by hybridizing with the other oligonucleotides. These DNA fragments now attached on both sides to the flow through the ligands undergo bridge amplification as shown in figures 8 and 9, leading to amplification of both the forward and reverse strand of the DNA fragment.

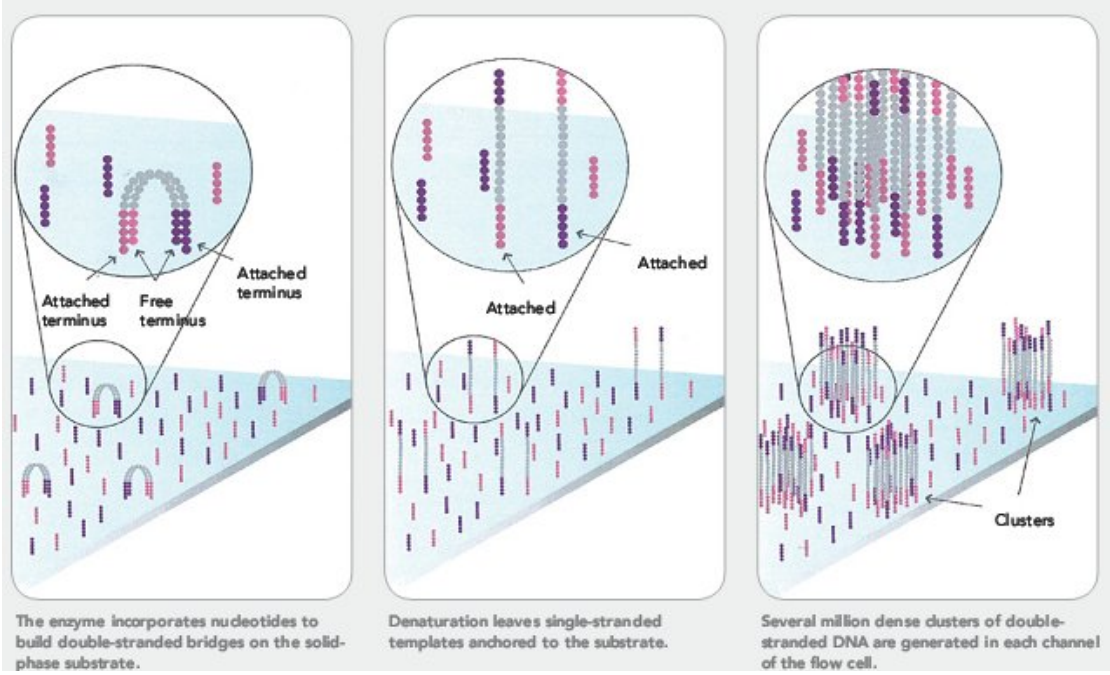


Figure 9. *Bridge Amplification and formation of read clusters. Retrieved from www.illumina.com*

The same process is followed through out all the lanes of the flow cell forming clusters of DNA fragments. Next the reverse strand is washed away and further amplification is blocked. The ligand present at the free end of the DNA fragment contains region that is complimentary to a sequences primer. Thus a sequencing primer is hybridized to the ligand that starts producing the read. Fluorescent nucleotides are attached to the DNA fragment producing a read. With each addition a light specific to that nucleotide is emitted from the flow cell cluster, depicting the nucleotide in the read. The number of time the light is emitted at a cluster depicts the length of the read. The intensity of the color emitted depicts the quality of the base call. This read depicting the read one is washed away. The DNA is bridged and a complementary sequence is produced washing away the first fragment. The sequencing process is again repeated, now in the opposite direction producing the read two. These reads are recorded on files known as fastq/fasta.

4.4.2 Alignment

The raw fastq files obtained from Illumina sequencing contains millions of reads in a raw state. Hence pre-processing needs to be performed to remove low quality reads. When the sequencer calls a base, it gives each base a phred score or quality score (q-score). The q-score denotes the probability that the based called could be an error. The minimum read quality is maintained as more than 20, as Kwon et al,(Kwon, Park, Lee, & Yoon, 2013) suggests that read quality less than this could indicate a 90% flawed base call. The pre-processing was performed using FASTX Toolkit, a toolkit (Blankenberg et al., 2010), and reads with quality less than 20 were removed.

Once the fastq files were filtered, the next step was to align them against a reference genome. The whole idea of aligning is to find the location of where a read completely aligns with the reference genome. This can be compared to a collection of sub-strings that needs to be matched to a bigger string. There are many algorithms available that can align the reads to the reference and can be run parallel using multi-threading. However, they require extensive memory capacity. Using Tries solves this problem. Also known as Prefix Tree or Radix Tree, a Trie is a data structure that can represent a given collection of sub-string in form of a tree. The basic idea behind the tries is to combine all the sub-string in form of a rooted tree, where sub-string flows in a 'from root to leaf 'path and each branch represents a letter of the sub-string. To further reduce the memory, suffix tries are used. All the possible suffixes of the entire genome are collected and taken created in the form of a trie.

A genome is a very large string, and if has to be indexed using the suffix tree, it would still take a lot of memory. This can be reduced; by encoding the genome, i.e. if there are repeats in the genome they are converted into runs. These runs that are both compressible and irreversible can be achieved using Burrows Wheelers Transform (BWT) (Figure 10).

4.4.3 Borrows Wheeler Transform

BWT is a reversible permutation of a string. The three crucial properties of BWT are that it is compressible, reversible and indexed. For a given string (here the genome), a symbol or a special character (here $) is added in the end of the string, to denote the position of the end or the star of the string once the string is permuted. All distinct rotations of the string are noted, i.e. all the possible permutations of the strings maintaining the same order are noted in the form of a matrix. Shown below is the cyclic rotations for banana$. The first column of this

matrix is sorted lexicographically and the resulting matrix is known as Burrows Wheeler matrix. From this matrix the string from the last column is known as Burrows Wheeler Transform.

BANANA$					**$B_1A_1N_1A_2N_2A_1$_1**
BANANA$		**$BANANA**	**A**	**$**	**$_1BANANA_1**
ANANA$B		**A$BANAN**	**N**	**A**	**A_1$BANAN_1**
NANA$BA		**ANA$BAN**	**N**	**A**	**A_2NA$BAN_2**
ANA$BAN	*Sorted*	**ANANA$B**	**B**	*Sorted* **A**	**A_3NANA$B_1**
NA$BANA		**BANANA$**	**$**	**B**	**B_1ANANA$_1**
A$BANAN		**NA$BANA**	**A**	**N**	**N_1A$BANA_2**
$BANANA		**NANA$BA**	**A**	**N**	**N_2ANA$BA_3**
BWT Matrix		BWT	Last Column	First column	First Last Property

From the last column the first column can be retrieved by lexographically sorting it. Then, the first and last columns when combined to form a two-dimensional matrix are known as 2mers. These 2mers when sorted can be used to re-construct (decompress) the whole original string in an order. The process of decompression is more efficient by a property of BWT, which is the 'First-Last Property'. From a Burrows Wheelers Matrix, for a particular symbol or character, its nth occurrence in the last column and nth occurrence in the first column, correspond to the same position in the original string. The statement can be re-arranged as, if a character 'a' has many repeats in a string, then the same character 'a' at a position 'x' in the string, has kth occurrence in both the first and second columns. For example, taking A_1, A2 and A_3 for instance. A1 in the last column and A1 in the first column belong to the same position in BANANA, that BA_1NANA. Similarly, A2 and A3 in the first column and last column belong to positions BANA_2NA and BANANA_3 respectively.

4.4.4 BWT Pattern Matching

Using the BWT and suffix array, the alignment of the read (sub-string) to the genome (string) can be performed efficiently with less memory. The genome is compressed to BWT. Combining this information with the first-last property, pattern matching can be achieved very efficiently. The tool Burrows Wheeler Aligner (BWA) that uses BWT, was used to perform the alignment of the filtered reads. The reads were aligned against canine reference assembly CanFam3.1. The output of the alignment in saved in a binary format in files known as Binary Alignment Map (BAM).

To identify the variations in the PGx genes from the bam files, the reads have to be processed using various tools, including BWA, Samtools, GATK, Picard, VCF-Tools and SnpEff.

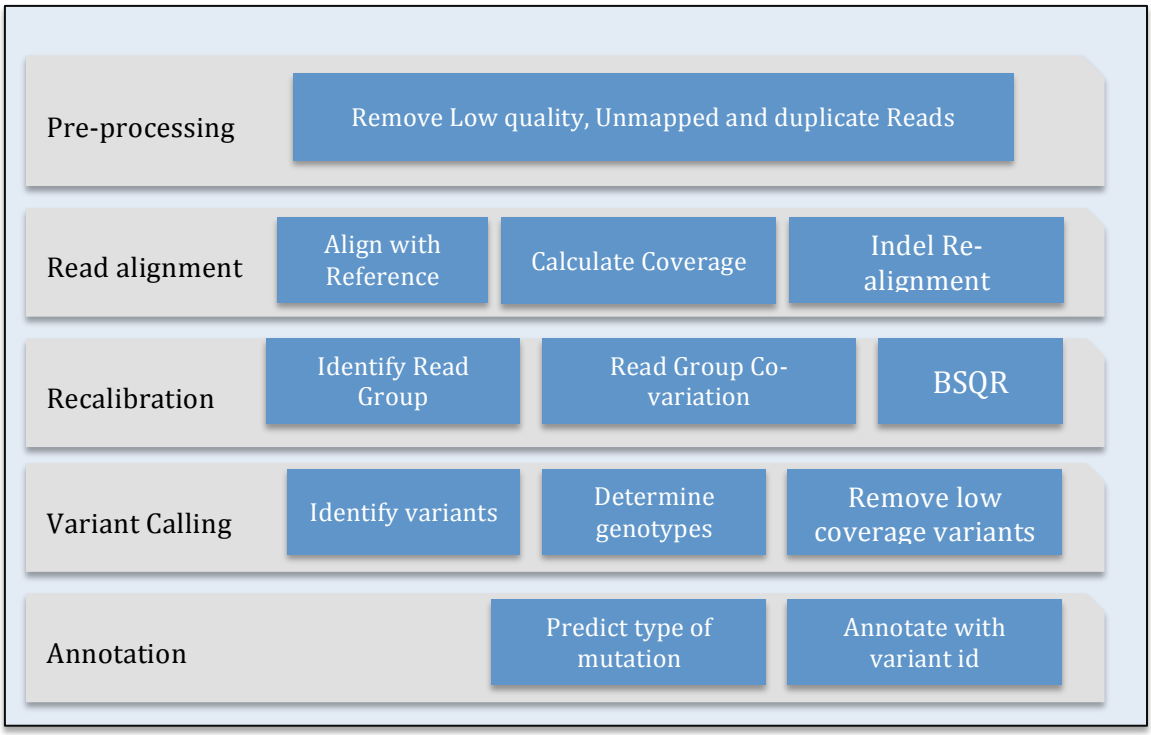


Figure 10. *An overview of different analysis steps of NGS sequence data to identify pathogenic variants.*

4.4.5 Pre-processing the aligned reads

Even after aligning the reads, a couple of pre-processing steps are needed. A Read produced by sequencer contains information of the location of the read in the first line and the nucleotide sequence in the next consecutive lines. Not every read produced by the sequencer needs to be mapped. Many reads could be left un-mapped due to various reasons such as poor quality or incompleteness of the reference genome or due to unknown genome contamination (Gouin et al., 2014). However, these reads can still map to the reference and might be a source of confusion or false discovery. Hence, these unmapped reads are removed before further analysis. In the process of sequencing, the DNA is amplified to make available enough samples for the sequencer to read the nucleotide sequence. This step results in duplicated reads. These reads are undesirable as they might impede the actual statistical proof of genotyping a variant such as allele frequency (Tin, Rheindt, Cros, & Mikheyev, 2014). In cases where many reads are duplicated with similar co-ordinates, the read that has better quality is retained and the others are removed. The unmapped reads were removed using Samtools (H. Li, 2011; H. Li et al., 2009). After some research it was analyzed that marking

the duplicates was better than removing them (Ebbert et al., 2016). Picard (http://broadinstitute.github.io/picard/) was used to mark the duplicate reads. We utilized here a PCR-free protocol for the WGS of 24 Border Collies to lower the number of 'PCR contaminants' in the data analysis and to improve the sequencing coverage elsewhere in the genome.

4.4.6 Calculate Coverage

Once the unwanted reads are removed, the remaining mapped reads are calculated for coverage. In theory, coverage can be mentioned as the number of times a nucleotide n a read is sequenced. The higher the coverage better is the quality of the sequencing and in an obvious manner higher is the cost. Though the overall or average coverage is given with a value, e.g. ~35x, it is possible that there are reads more than 90x and some even less than 10x. When coverage of some portion of genome is low, this can affect the reliability of the variant calling. For instance, if there are only total of 4 reads for a locus and 2 reads among them were called wrong due to some sequencing error, then this leads to call of a false variant. Thus, it is always a good practice to calculate the coverage (Sims, Sudbery, Ilott, Heger, & Ponting, 2014). The overall and genomic region specific coverage is calculated using qualimap (Garc??a-Alcalde et al., 2012).

4.4.7 Indel Re-alignment

The insertions or deletions in a read sequence can be confusing for the aligning algorithm to align with the reference. The Indels could be easily misinterpreted as multiple SNPs and can also disturb the recalibration process. Local re-alignment around target intervals is quite essential to avoid false discovery of SNP. Local re-alignment looks for problem causing regions, where there could be a possible indel. Multiple consecutive SNPs in a read are one such possible locus. In such locus, the reads are re-aligned by finding the possible alternate consensus sequences. The consensus sequence is scored, by summing up the mismatch scores. The alternate consensus sequence with the best score is selected rather than the original alignment. The indel realignment is performed using 'IndelRealigner' option from GATK (DePristo et al., 2011).

4.4.8 Re-calibration

Each single base has a phred score that depicts the quality of base in a read. However, the score of a single base is not sufficient to determine the quality of the read. Reads that are run

together in a batch in a sequencing machine belong to a read group. Each read contains a read group id, depicts the read group it belongs to in the sequencing machine run. Though the phred scores can depict the error probability of a single base call, it is not appropriate measure to identify insertions or deletions. For this covariates are calculated between the phred score of the base in a read, the position of the base in the read, the previous nucleotide and it's phred score and the machine cycle the base is produced. The recalibration of a base is done in two steps. The first step includes, creation of recalibration table and using this data the bam is re-calibrated in the second step. The re-calibration table contains the details of the number of bases in a read group and the frequency of mismatched bases, as per dbSNP. Once the recalibration table is created, the quality scores of the reads in the old bam file are re-calculated and written to a new re-calibrated bam file. These new phred scores are essential to identify indels (DePristo et al., 2011). Using dbSNP data from DoGSD as a reference dbSNP, recalibration was performed using 'BaseRecalibrator' option from GATK (Bai et al., 2015).

4.4.9 Variant Identification

From the bam file, once the reads are aligned with the reference and re-calibrated, the single nucleotide bases that differ from the reference are identified as SNPs (Nielsen et al., 2011). The nucleotide from the aligned reads at each genomic position is first genotyped and variants are identified based on the genotype information. The base call intensities from the sequencing are noted in terms of per base quality score based on noise from image analysis. This values is converted to phred score by the below formula

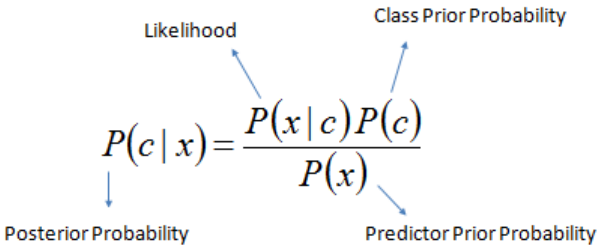
$$Qphred = -10\log10\ (error)$$

(Qphred = 20 => 1% error)

Formula.1. Calculation of phred score

As this cannot be trusted completely, recalibration was performed using GATK using the empirical phred scores that was calculated as the difference between the mismatches between the base call and the reference genome and the mismatches implied by the raw quality score. This empirical phred score is added to the raw quality score to obtain the recalibrated quality scores. These base calls and the recalibrated quality scores are used to determine the genotype and eventually the variant. In the classic method of genotyping, the number of alleles at a site is counted and would be determined heterozygous if the non-reference allele is between 20-

80%; else it is determined as homozygous depending on weather the allele is reference or non-reference. However, this could work as described only if the coverage is about 20x and this method also does not provide the probability of certainty of the genotype called. Hence the best available option for genotype calling is probabilistic models. The genotype calling using probabilistic models are done using Bayesian probability.



Formula.2. Bayesian probability explained

Applying the Bayes Theorem to calculate the posterior probability P (G|X) of a genotype G it is essential to calculate the genotype likelihood and the prior probability. The quality scores of each read are used to calculate the genotype likelihood P(X | G). If the number of reads at a particular site is given by i, then the genotype likelihood is estimated as

$$\mathbf{P(X \mid G) = \Pi_i P(X_i \mid G)}$$

where $P(X_i \mid G)$ is quality score of X_i (data in read i)

Formula.3.Genotype Likelihood based on Bayesian probability theorem

The prior genotype probability P (G) for each genotype is either assigned equally or by using information from external databases such as dbSNP.

The variants identified are output in a Variant Call Format (VCF) file. GATK and Samtools are the tools used to identify the SNPs. Most of the Indels could be misinterpreted as SNPs. The earlier step indel re-alignment avoids the False Discovery Rate (FDR) of SNPs. Indels are identified using tools GATK and Samtools. To ensure good quality predictions variants that have a minimum depth of 10 and have a minimum quality score of 40 were selected. The variants identified by different tools were combined using the GATK 'CombineVariants' option and written to a VCF output.

Variant databases have a broad set of SNPs, where each variant is tagged with a variant identifier. The GATK variant annotator, marks these ids to the variants in the filtered vcf file, i.e. if a variant present in the filtered VCF file has an id tagged to it in the database, then the annotator, tags the id to the variant. These ids were added to the Id Field in the VCF File.

4.5 **Filtering PGx variants**

At this stage, the two parallel segments of the pipeline were completed: (1) create bed files with ORF genomic positions of the PGx genes; (2) identify variants from the samples under study. The next step was to combine the outputs of the two segments and filter the variants based on the ORF genomic positions of PGx genes. Thus, a two-step filtering was performed. One, only variants related to PGx genes were retained and two, only variants that belonged to the ORFs were selected. Also variants from psuedogenes were filtered out. The remaining variants from all the samples under study were combined into a single file using GATK tool.

4.6 **Pathogenicity Prediction**

Proteins that have the same function in different species (orthologues) have evolutionarily conserved sequence structure especially in the functional domains. In such conserved positions, when the existing amino acid is changed by another amino acid with different properties, the change is potentially deleterious.

Two programs, SIFT and PolyPhen 2, are used to predict the pathogenicity of the variants based on evolutionary conservation. SIFT gets sequences from all the available protein databases and aligns it using PSI- BLAST to get a homologous sequence. It aligns the query sequence with the homologous sequence to create a scaled-probability matrix. The below figure is an example of scaled probability matrix. The matrix contains the calculated probability for all possible 20 amino acids at a position and normalized with the probability of the most frequent amino acid at that position. This probability is also known as scaled probability, which is defined using the below formula.

$$p_{ca} = \frac{N_c}{(N_c + B_c)} * g_{ca} + \frac{B_c}{(N_c + B_c)} * f_{ca}$$

Formula 4. Scaled Probability P_{ca} for amino acid ‘a’ at position ‘c’ (Ng & Henikoff, 2001)

Where

N_c = Number of amino acids in the sequence

B_c = Number of pseudo counts exp ($\Sigma_a(r_a * g_{ca})$)

r_a =rank of the amino acid a in the BLOSUM matrix

g_{ca}= sequence weighed frequency that amino acid a appears at c (for normalizing)

f_{ca} = Pseudo count function added to N_c

A substitution is considered deleterious if its score is below a threshold (here < 0.05) (Ng & Henikoff, 2006).

pos	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
1M 0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2E 0.25	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3M 0.50	0.07	0.02	0.02	0.03	0.12	0.02	0.02	0.24	0.03	1.00	0.63	0.02	0.03	0.03	0.03	0.03	0.05	0.17	0.02	0.04
4A 0.50	1.00	0.05	0.13	0.17	0.04	0.68	0.04	0.05	0.16	0.09	0.04	0.12	0.14	0.10	0.10	0.30	0.15	0.11	0.01	0.05
5C 0.75	0.59	1.00	0.17	0.15	0.09	0.33	0.08	0.11	0.17	0.15	0.06	0.19	0.18	0.12	0.12	0.93	0.44	0.21	0.03	0.11
6R 0.75	0.37	0.04	0.24	0.36	0.06	0.23	0.11	0.10	0.58	0.17	0.06	0.26	0.15	0.29	1.00	0.63	0.33	0.15	0.02	0.09
7V 0.75	0.10	0.03	0.02	0.03	0.22	0.04	0.03	0.99	0.04	0.48	0.10	0.03	0.03	0.03	0.04	0.05	0.09	1.00	0.02	0.09

Figure11. *Scaled Probability matrix for a protein sequence (Ng & Henikoff, 2006)*

Polyphen 2, aligns the proteins from closely related species using MSA. From the MSA, Position Specific Independent Counts score is calculated (PSIC Score), which represents the logarithmic ratio of likelihood of a particular amino acid occurring at a particular position to the likelihood of occurring at any other position (background frequency). These scores are accumulated to form the profile matrix for a protein sequence. For a mutation, the difference in the PSIC score of the reference and the mutant is calculated as ΔPSIC. Very high ΔPSIC value indicates a possibility of pathogenic mutation(Adzhubei, Jordan, & Sunyaev, 2013). Polyphen2 also uses a structure-based prediction along with the sequence-based features to predict the pathogenicity. It gathers structure related protein information from databases like Dictionary of Secondary Structure in Proteins (DSSP), Protein Data Bank (PBD) and also calculates this information based on some protein structure parameters (such as hydrophobicity, electrostatic interactions etc.). It maps the amino acid change to the structure information available to decide if the amino acid is pathogenic(Adzhubei et al., 2013).

The final VCF file that was created by combing all the variants was first annotated using SnpEff tool. SnpEff is a variant annotation tools, that interprets the variants based on the chromosomal position and predicts the possible effect based on available information such as transcript information, protein sequence, etc. (Cingolani et al., 2012). Some of the common effects predicted by the SnpEff includes Synonymous variant, Non-Synonymous variant, frame shift, stop gain/stop lost codon. As Polyphen2 and SIFT performs predictions only for SNPs for a canine genome, pathogen prediction was performed separately for Indels. SnpSift was used to select the variants with specified effect predictions. Non-synonymous variants were selected from SNPs, frame-shift from indels, stop-lost and stop-gain variants were selected.

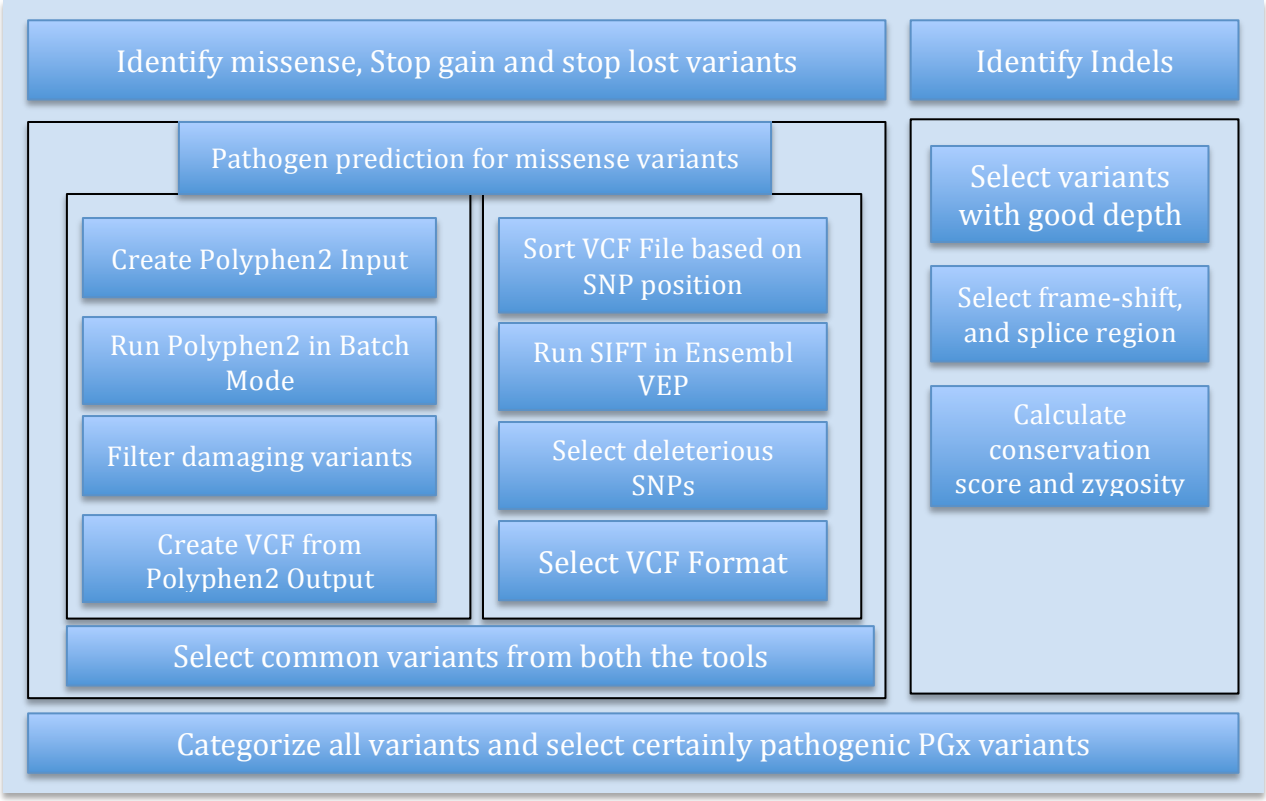


Figure 12. *Pathogen Prediction pipeline for Indels and SNPs.*

While SIFT takes VCF as an input; Polyphen 2 requires an input in a specific format. Thus, VCF is given as input to SIFT and a formatted text file for Polyphen2. For Polyphen 2 the input files are run in array batch mode as described in the manual to reduce the time required for the process. From the output only the variants that are tagged as 'probably damaging' and have a Bayesian probability greater than 0.98 are retained. Similarly SIFT is run from Ensembl variant effect prediction (VEP) tool using the default parameters. Here only the variants that are identified as low tolerated, highly confident and have a SIFT score greater

than 0.9 are included. The filtered variants from SIFT and Polyohen2 are further filtered to retain only the SNPs predicted pathogenic by both the tools.

Unlike for the SNPs there are rather few tools to predict the effect of Indels for Canine genome. Hence they need to be confirmed using genome viewer tools such as IGV. As it is difficult to view so many Indels, a two-step filtering is used. From these indels only frame-shift and splice region variants are selected, as these are the most probable indels that can be deleterious. Next, only the variants with a depth greater than 20 are kept. Another interesting feature to be observed here is breed specific variants. Variants in a homozygous state in all the samples could either be variants specific to Border collie or Boxer (the reference genome breed) and hence are removed. The remaining set of mutations is mostly true and deleterious, however they are confirmed using IGV.

The missense variants identified as pathogenic by both SIFT and Polyphen2, the stop mutations and the frame-shifts selected from the above processes are combined to get the final list of interesting variants, among the 24 samples included in this study. To increase the pathogenicity confidence of the indels the allele frequency and zygosity of the variant are calculated using genotype frequency. Based on the information calculated, the variants are categorized into pathogenic with high confidence, likely pathogenic and unlikely pathogenic.

4.7 **Known functional inference**

There are many clinical or disease or drug-related databases that provide implication of many known mutations. The only online database for Canine is Online Mendelian Inheritance for Animals (OMIA). However, there are many clinical or disease related database for human such as CliniVar, Online Mendelian Inheritance for Man (OMIM), PharmGKB. To be able to use the information from human databases, the genomic co-ordinates of certainly pathogenic variants are converted to its orthologuous base in human genome using liftOver. LiftOver is an online tool developed by the University of California, Santa Cruz (UCSC) genome browser, and it provides amino acid in the human reference orthologous to the one at the canine reference. The genomic positions are converted from CanFam3 to hg38.

To understand the biological characteristic of the certainly pathogenic variants, functional analysis is performed using DAVID online tool.

5. Results

5.1 Identification of Canine PGx genes

To generate the list of Canine PGx genes, we first developed a list of known human PGx genes from various sources, including PharmGKB, PharmaADME databases and the gene list (382 genes) from prof. Mikko Niemi at the Department of clinical pharmacology. The list of genes includes 47 genes from PharmGKB VIP gene set and 200 and 110 genes from PharmGKB CPIC and drug related genes set, respectively. From the PharmaADME database, 31 Core genes, 267 extended and 74 related PGx genes were selected. The total set included 540 genes and they were divided into three categories: core genes, extended genes and related genes (Figure 13). The core genes consisted of the genes that belonged to VIP gene set from PharmGKB and core gene set from pharmaADME. The extended gene set consisted of genes with known drug information (genes that belonged to drug related gene set) from PharmGKB and the genes that belonged to extended gene set from pharmaADME. The related genes were the genes that belonged to the related category (target/receptor genes) from pharmaADME and other genes that do not belong to any other categories.

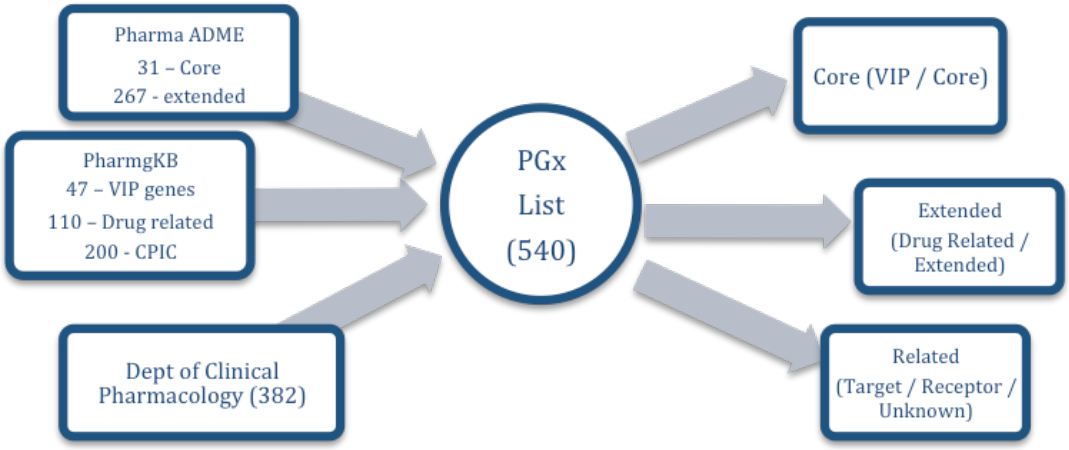
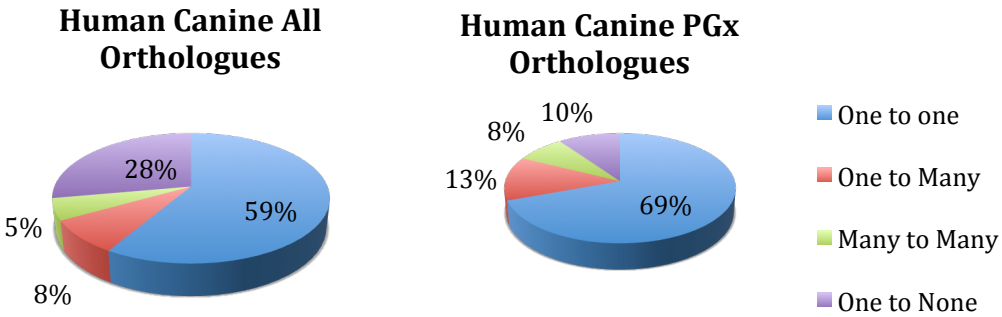


Figure 13. *The generation of the human PGx list included in further studies in the pipeline.*

Canine orthologues were identified using the Ensembl Compara API tool. We retrieved 495 orthologous canine genes for 492 human genes. Among the 495 orthologues, 60 were likely pseudo-genes genes. Pseudo-genes are said to be the genes, that are evolutionarily related to functional protein coding genes, but are suspected to have lost the protein coding functionality due to various possible mutations such as a frame-shift or stop mutation. Even though these 60 genes are pseudo-genes, recent studies using high-throughput technologies

have suggested that pseudo-genes could possess gene-expression regulatory functions (W. Li, Yang, & Wang, 2013). There are also studies that suggest pseudo-genes have important associations with pharmacogenomic genes and also act as therapeutic targets in some cases(Roberts & Morris, 2013)(Cordero & Ashley, 2012).

Most of the orthologue pairs are mapped one to one, i.e one human gene to one canine gene. For example, human ABCB1 and TPMT have MDR1 and TPMT as canine orthologues (only one orthologue each). However, there were few orthologue pairs on an one-to-may-many basis. For example, the canine CYP2A13 gene mapped to three human genes CYP2A13, CYP2A7, and CYP2A6. The likely reason for this is that the three human genes are paralogs to each other with high sequence similarity. However, the most likely orthologue pair can be checked from the e-value (appendix Table 1) (Figure 15). Including both on-to-one and one-to-many orthologue pairs, a total number of 532 human-canine orthologue pairs were observed (with 492 human and 495 canine genes, Figure 14).



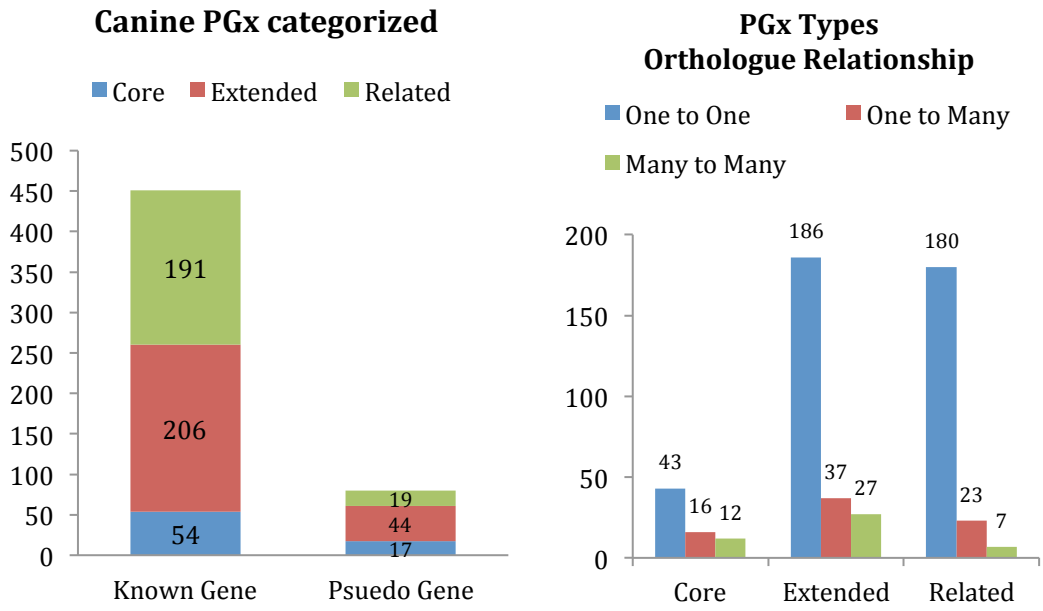


Figure 14. *a) Categorizing all the human and canine orthologues based on type of orthologue relationship b) Categorizing PGx genes based on type of orthologue relationship. c,d) Categorizing the PGx genes based on PGx type(Core, Extended and Related)*

Chi squared test with Yates correction was performed to check how enriched the canine PGx list was with genes that have a one to one orthologue relationship with human genes.

Table 3. Contingency table for the fischer exact test. The pValue from the fischer.test was about 0.001

	Other Relationship	One to one
Other Genes	11021	15398
PGX Genes	180	409

To understand the protein sequence similarities between the orthologues, eValues from pBlast were obtained for two orthologue peptides from Ensembl compara API. When an eValue is very small (negative exponentials) it is close to zero. For the convenience of understanding, such values are converted to the minimum evaluable scientific number, which is '2.225074e-308'. Also the number of base pairs in open reading frame of the orthologues was retrieved.

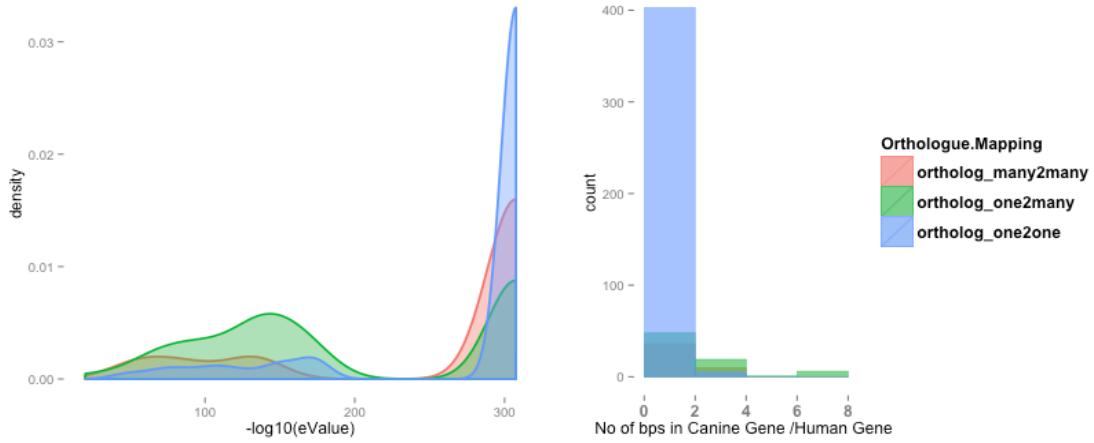


Figure 15. *a) A density plot depicting the distribution of negative log10 e-value of the orthologue peptides with different types of orthologue mapping. b) Histogram of then ratio of the number of canine base pairs with the number of human base pairs.*

5.2 PGx Annotation Table

A category annotation table was created using the information from PharmgKB and PharmADME databases. The table contains information for each PGx gene in human; it's canine orthologue, the type of mapping (one2one, one2many and many2many), the gene type (core, extended or related) and it's pharmacogenomic role (enzyme, transporter, receptor,

modifier etc). Based on pharmacogenomic importance, the genes are categorized into core, extended or related as described in the human Pharmacogenomic genes section. Based on their known pharmacogenomic function they were categorized as transporter, modifier, Phase I enzyme, Phase II enzyme, target or a receptor. If it did not belong to any of the categories then they were marked as 'unknown', so that it could be updated once the information is available. The last category was based on its orthologues mapping with human as described in the above section into one2one, one2many or many2many orthologues mapping. With these information collected from PharmgKb, pharmaADME and ensembl an annotation table was created to assist in further downstream analysis. An example of the annotation table for the orthologue genes is shown in the example table below. The complete table is available in the appendix (Table 1).

Table 4. An example of PGx annotation table

Human Gene	Importance	Type	Canine Gene	Orthologue Mapping
ABCA1	Transporter	Extended	ABCA1	one2one
ABCG2	Transporter	Core	ABCG2	one2one
ADH5	Phase-I	Extended	ADH5	one2many
HMGCR	Target/Receptor	Core	HMGCR	one2one
AHR	Modifier	Core	AHR	one2one

5.3 NGS Data Analysis

The output of the Illumina sequencing is in the fastq file form, for all the twenty-four Border collie samples.

5.3.1 Remove reads with low quality reads

The initial step of the pipeline is to pre-process the reads to remove bad quality and unwanted reads. In the first step the reads were trimmed to remove the low quality reads by using a threshold quality score 20. Eventually all the reads less than the quality score 20 were filtered out. For the convenience of representation one of the samples is selected to report the statistics of the NGS analysis (BC223). After this step 97.99 percent of the reads passed the quality filter that is used for the next processing step while 2.01 percent of the reads were removed.

5.3.2 Align with reference and coverage

The fastq files from the above step were aligned with the reference genome CanFam3.1. The coverage of all the samples was calculated (Figure 16).

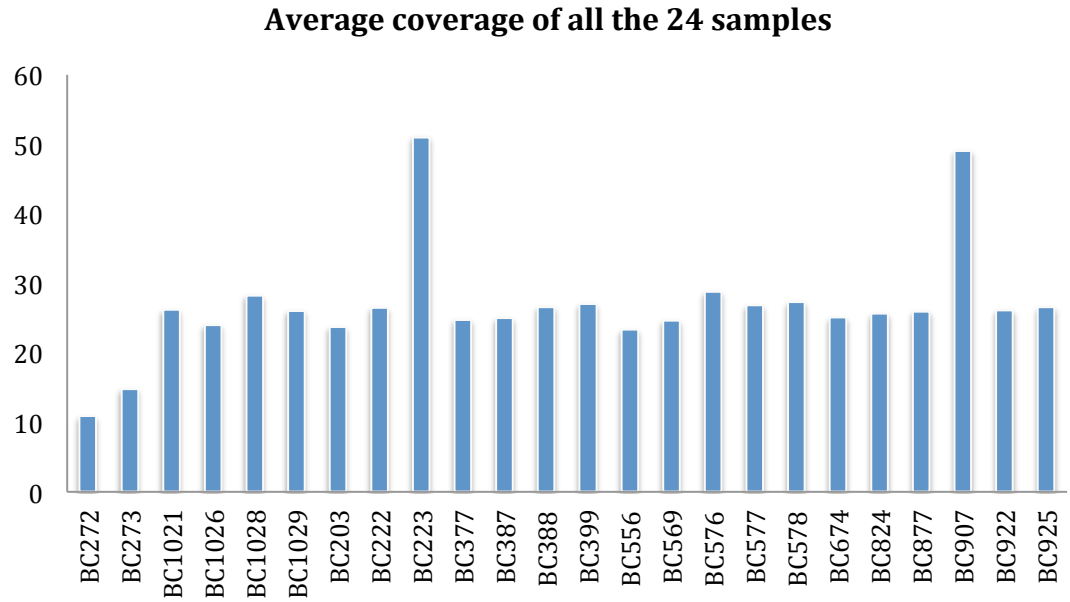


Figure 16. *A plot representing the coverage after pre-processing the low quality reads. The X-axis and Y- axis represents the 24 samples and the average coverage across the whole genome.*

After the alignment the reads that were left unmapped were removed. 0.6 % of the reads was found to left be unmapped and were removed. From this output of this file duplicate reads (formed due to PCR amplification) were removed. About 3.39 percent of the reads were removed in this step. The output bam after this was a successfully pre-processed file. The pre-processed bam file was re-calibrated using dbSNP data from DoGSG as a reference. The statistics of all these steps are presented in the tables below (Tables 4-7)

Table 5. Statistics of reads before alignment

	Raw fastq Files	% of Total Reads	Filtered Fastq file	% of reads passed	% of Reads removed
Read 1	658,200,600	51.25	643,444,600	97.76	2.24
Read 2	626,093,375	48.75	615,667,265	98.22	1.78
Total Reads	1,284,294,105	100	1,259,111,865	97.99	2.01

Table 6. Statistics of reads aligned to reference

Initial Reads	**Mapped Reads**	**UnMapped Reads**	**% Mapped Reads**	**% Unmapped Reads**
1,259,111,865	1,250,549,904	8,561,961	99.324	0.676

Table 7. Statistics after marking the duplicated reads

Before Marking Duplicates	After marking Reads	Marked Reads	% Marked Reads
1,250,549,904	1,209,546,285	410,003,619	3.39

Table 8. Statistics after re-calibration

Total number of reads	Reads from 1st in pair (Number and %)		Reads from second in pair (Number and %)	
1,209,546,285	606,635,506	50.15	602,910,779	49.85

The chromosome wise coverage was calculated after re-calibration for a better insight into the coverage details.

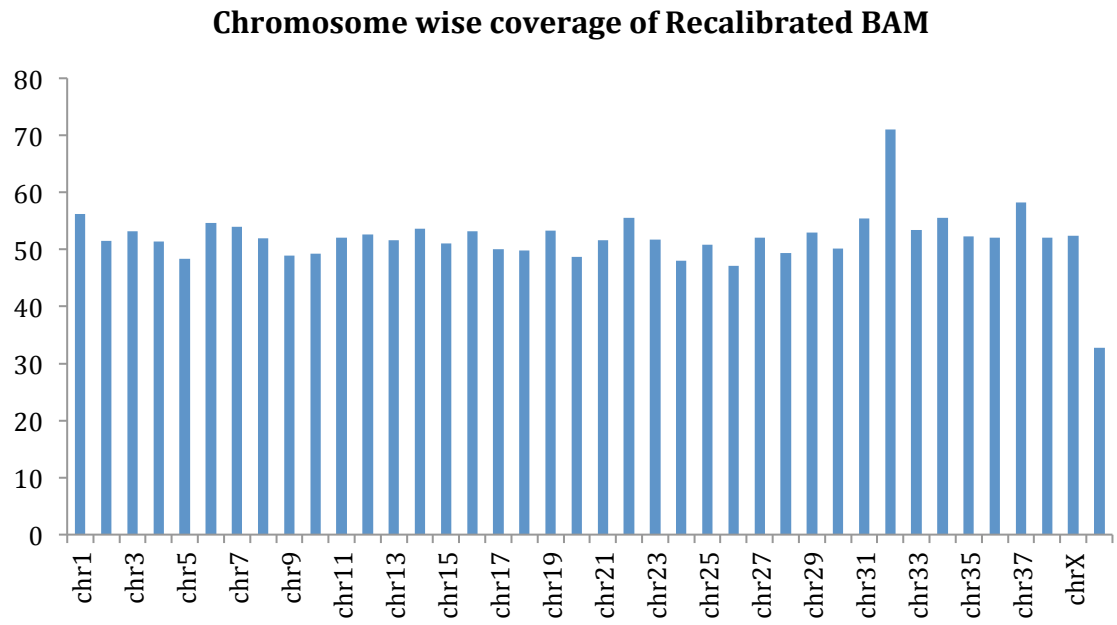


Figure 17. *Plot representing chromosome wise coverage.*

5.4 Variant Identification

The final recalibrated bam contains 94.17 % of reads compared to the raw reads after the pre-processing and recalibration steps. These reads were used to call the variants that include SNPs, Indels and some structural variants.

With the default set parameters GATK identified 4771681 SNPS and 1391674 Indels while Samtools identified 4595077 SNPS and 189339 Indels. The variants were combined based on the chromosome position and the nucleotide change in the variation using the GATK combine variants option. Both the tools identified 96.09 % of SNPs. GATK and Samtools individually identified 2.23 % and 1.68% of the SNPs. Similarly, out of the 1581013 total potential indels identified, 550812 indels that contribute to 65.16% from the combined lists were the ones

identified by both GATK and Samtools. GATK and Samtools separately identified 361473 and 189339 indels that respectively represents 22.8 % and 11.9 % of the final list of potential indels. The table represents the statistics of SNPs and INDELS identified by GATK and Samtools.

Table 9. Statistics of SNPs identified.

	SNPs by GATK	Only by GATK	SNPs by Samtools	Only by Samtools	Common by both tools	Total SNPs
Number	4771681	16604	4765424	10347	4595077	4782028
Percentage	99.7	2.23	99.65	1.68	96.12	100

Table 10. Statistics of Indels identified by GATK and Samtools.

	Indels by GATK	Only By GATK	Indels by Samtools	Only by Samtools	Common by both tools	Total Indels
Number	1391674	361473	1219540	189339	550812	1581013
Percentage	88.02	22.8	77.13	11.9	65.16	100

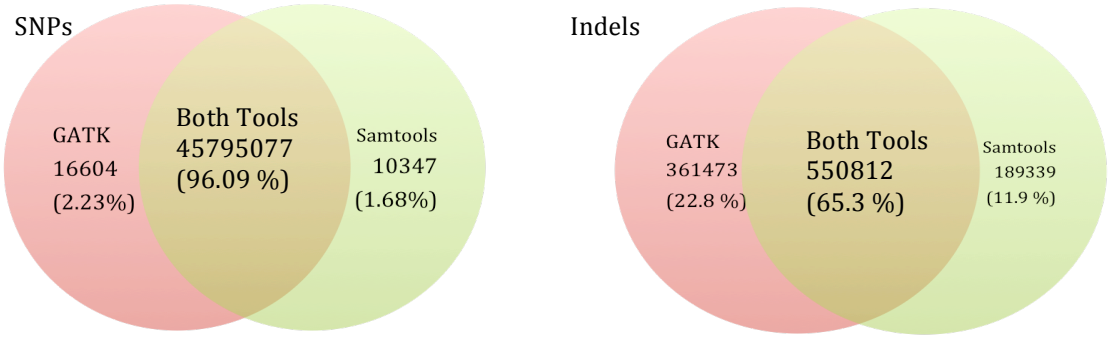


Figure 18. *Venn Diagram depicting the proportion of variants identified by different too*

5.5 Variant Filtering

The final variants from the NGS pipeline were further filtered through a series of steps to retrieve the final list of predicted pathogenic variants in PGx genes. The length of the Canine genome is about 2.3 Gbp while the length of the pharmacogenetic genes approximates to 0.022 Gbp or 22.03 Mbp that comprises of about 0,1 % of the whole genome. Hence first step was to select only the variants from these genes, to reduce the disk space and computation time for the next processing steps. From the below table it can also be observed that the open reading frames of the PGx genes comprises about 40% of the whole genes. As this study was mainly concentrated on the coding regions, only the variants that occur in open reading frames (ORFs) were selected to further reduce the computational time and disk space. A bed

file was created using Bedtools with information of genomic regions of just the ORF of PGx genes. This bed file was used to filter the variants. The final output of this step is also a VCF file.

Table11. Length of Canine PGx genes and ORFs. Length in Base Pairs

Whole Canine Genome	Pharmacogenomic (PGx) Genes	PGx Genes ORF
2,392,715,236	22,031,443	922,260

The VCF file from the above step was then annotated with SnpEff and ensembl annotations. From the annotation, variants that were missense, frame-shift, Stop-Gain or Stop Lost and splice site region variants were selected, as these are the types of mutations that are usually pathogenic. The numbers of mutations in each category are depicted in the flowchart below.

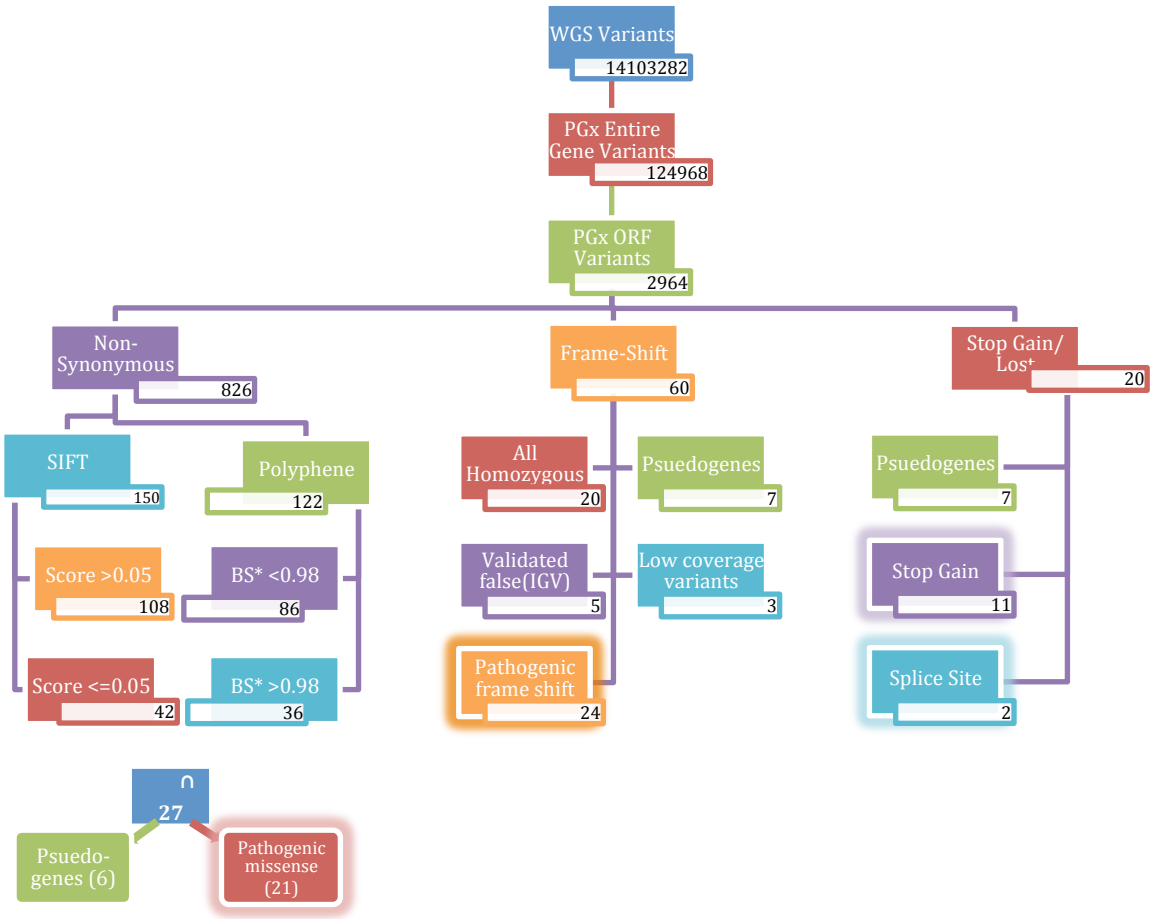


Figure19. *Number of variants at various stages of filtering from all the 24 Border Collie samples.*

5.6 **Pathogen variation prediction**

The Missense variants were further processed to find or predict the possibly pathogenic mutations. Polyphen2 classifies the predictions into benign, possibly damaging and probably damaging. The variants that were predicted probably damaging with a Bayesian probability greater than 0.95 are selected, as they are the one with high confidence. SIFT, identifies the pathogenic variants or deleterious variants and gives a confidence score. The variants that were deleterious with high confidence were selected. As Polyphen predicts based on sequence alignments and SIFT based on conservation score, selecting only the variants that are predicted pathogenic by both the programs, further increases the confidence of the prediction. Among the 826 variants from 24 samples, 42 variants from Polyphen2 and and 36 variants form SIFT were selected based on their respective selecting criteria as mentioned above.

Table 12. Variants identified by Polyphen 2

Predicted as benign	Predicted as damaging	Predicted as damaging with Bayesian score > 0.95 (selected)	Predicted damaging based on alignment	Predicted damaging based on structure
704	122	42	40	2

Table13.Variants identified by SIFT.

Predicted benign	Predicted deleterious	Deleterious with high confidence
676	150	42

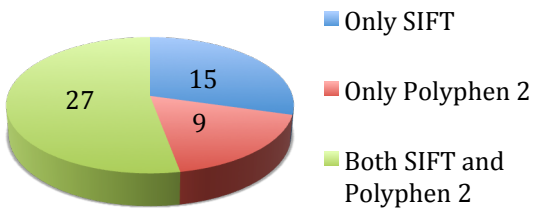


Figure 20. *Pie diagram depicting the overlap between Polyphen2 and SIFT*

The indels, that included frame shift, stop gain / stop lost and splice site mutations were further analyzed in IGV. Variants that were false positive and had very low coverage were removed. An example of low coverage is depicted in figure 21 at 'chr7:79047553:GCCCC>GCCC', the coverage is very less and hence the genotype cannot be reliable.

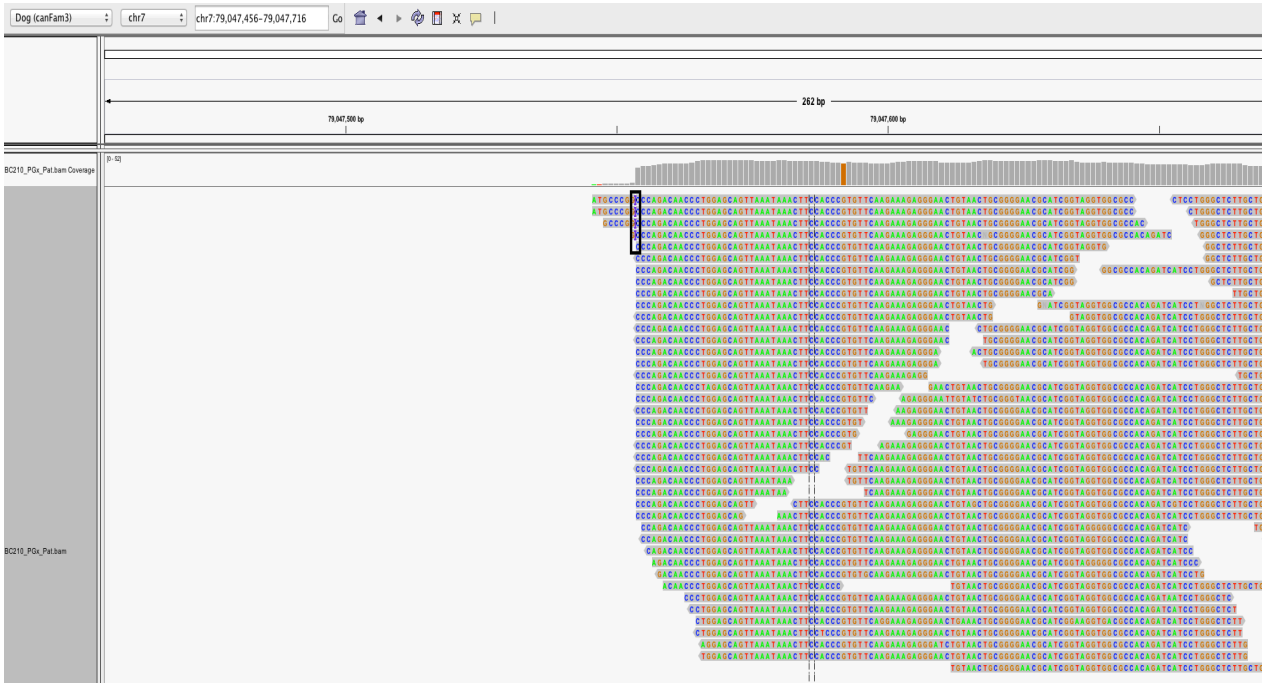


Figure 21. *IGV screen shot of a variant filtered out due to low coverage*

One of the indels identified at chromosome position ‘ is a false positive as depicted in figure 22. It can be seen that the indel is actually two snps at adjacent positions and is falsely called as an indel. From the frame-shift variants 2 and 3 variants were removed due to low coverage and false positives respectively.

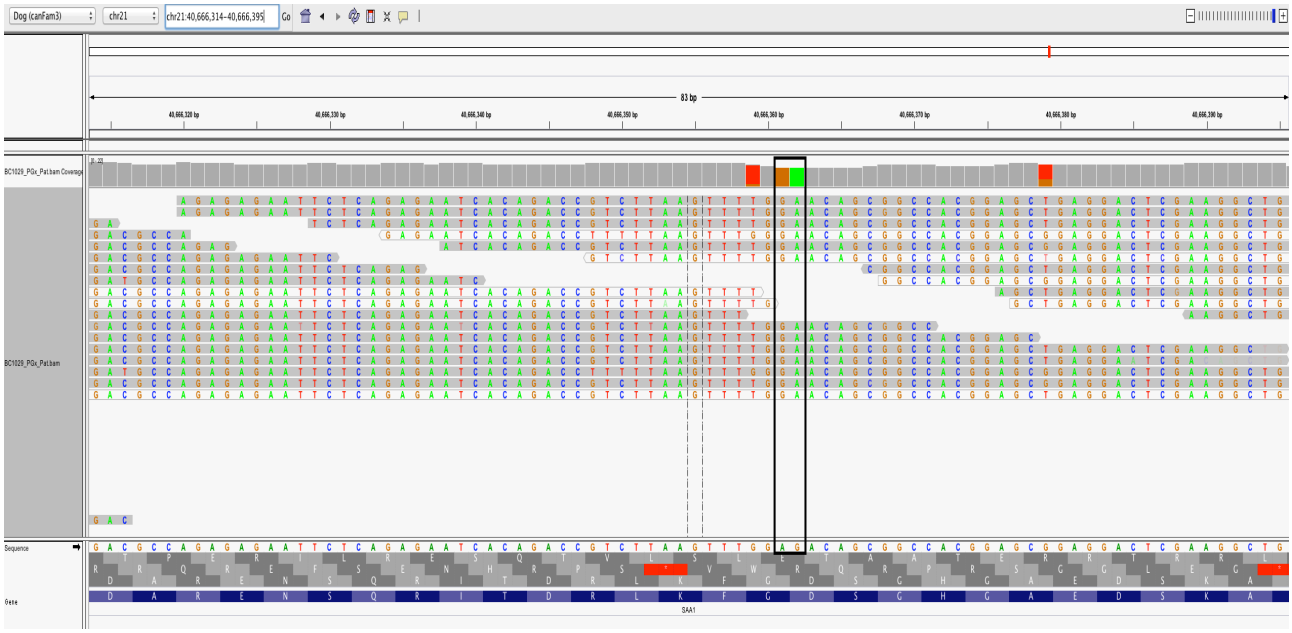


Figure 22: *IGV screen shot of two consecutive snps false called as an indel.*

A simple algorithm was written to calculate the number of homozygous and heterozygous mutation at each site and the allele frequency based on the genotype frequency. In the final step, 20 variants were removed, as they were present in all the samples in a homozygous state implying that they could be specific to Border collie or the Boxer (reference genome). All the remaining variants were then categorized into three classes such as pathogenic variants with high confidence, likely pathogenic and unlikely pathogenic. Highly confident pathogenic variants included the frame-shift and stop-gain indels after the filtering and the missense variants identified by both SIFT and Polyphen 2. The highlighted boxes in figure 19 represent the set of highly confident pathogenic variants. Likely pathogenic variants include breed specific variants, variants with low coverage and pathogenic missense variants identified by either SIFT or polyphen2. The variants that did not come under any of this category belonged to unlikely pathogenic variants.

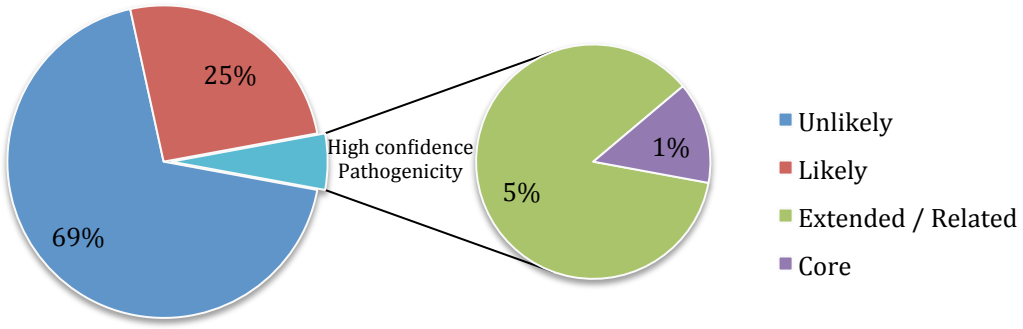


Figure 23. *Illustration of a pie chart; depicting the number of variants, at each level of pathogenicity.*

A total of 68 variants were classified as certainly pathogenic variants out of which 9 belonged to core genes and 59 belonged to either extended or related genes. Among the rest of the variants 251 variants belonged to the likely pathogenic category while 657 variants were categorized as unlikely pathogenic variants. The complete list of pathogenic variants with high confidence from this study is listed here below (Table 14).

Table 14.1: The final list of missense pathogenic variants with high confidence and their PGx information.

Gene	CHR	POS	REF	ALT	PGx Imp	Function	AA Change	0/1	1/1
ABCB4	chr14	13543365	C	T	Extended	Transporter	p.R1191G	4	0
ABCC10	chr12	11915224	T	G	Extended	Transporter	p.A1120S	9	2
ABCC11	chr2	66872504	G	A	Extended	Transporter	p.N1099S	3	0
ABCC8	chr21	39963480	C	T	Extended	Transporter	p.S1485G	5	4
ACAA2	chr7	79043218	A	C	Related	Unknown	p.S189Y	1	0
CRAT	chr9	54576878	A	G	Reltated	Unknown	p.E388K	1	0
CYP2J2	chr5	49978117	A	G	Core	Phase-I	p.A407T	2	0
DHRSX	chrX	1190103	T	C	Extended	Phase-I	p.A161T	8	3
DLA-DQA	chr12	2225877	A	G	Related	Unknown	p.V221M	9	0
DLA-DQA	chr12	2225964	T	C	Related	Unknown	p.L250F	4	18
EPHX1	chr7	38980909	T	C	Extended	Phase-I	p.R71H	7	0
FMO3	chr7	27725328	A	G	Extended	Phase-I	p.P358S	1	0
FMO6P	chr7	27686759	T	C	Extended	Phase-I	p.G442R	3	2
MDR1	chr14	13710943	G	T	Core	Transporter	p.Q197H	4	0
PML	chr30	37265646	T	C	Related	Unknown	p.R457W	3	0
RPS6KB1	chr9	34424229	T	C	Related	Unknown	p.P448S	5	1
SLC22A1	chr1	49277508	A	G	Core	Transporter	p.G218D	11	4
SLC22A1	chr1	49279319	A	G	Core	Transporter	p.G308E	5	0
SLC22A10	chr18	53608679	G	T	Extended	Transporter	p.S189R	7	3
SLC22A12	chr18	52549442	A	G	Extended	Transporter	p.L72F	9	0

Table 14.2: The final list of Stop gain /splice site pathogenic variants with high confidence and their PGx information.

Gene	CHR	POS	REF	ALT	PGx Imp	Function	AA Change	0/1	1/1
ALDH3B1	chr18	49807490	G	A	Extended	Phase-I	R198*	3	0
ALDH3B1	chr18	49807373	C	A	Extended	Phase-I	E237*	2	1
ALDH3B1	chr18	49807860	A	T,C	Extended	Phase-I	C74*	3	17
ALDH3B1	chr18	49807979	G	A	Extended	Phase-I	Q35*	3	19
AOH2	chr37	9870736	C	T	Extended	Phase-I	R518*	2	0
CYP1A2	chr30	37821686	C	T	Core	Phase-I	R373*	7	0
CYP3A26	chr6	9797643	C	A	Extended	Phase-I	E122*	3	0
DLA-DRB1	chr12	2157139	G	C	Related	Unknown	Y119*	1	0
DLA-DRB1	chr12	2157346	G	T,C	Related	Unknown	Y50*	9	2
SAA1	chr21	40704199	G	T	Related	Unknown	G93*	1	0
SAA1	chr21	40684958	G	T	Related	Unknown	G109*	1	0
SLC22A18	chr18	46885448	GG	GGGTG	Extended	Transporter	.	0	1
SLC22A18	chr18	46885444	GGG	GGGGGG	Extended	Transporter	.	7	14

Table 14.3: The final list of frame-shift pathogenic variants with high confidence and their PGx information.

Gene	CHR	POS	REF	ALT	PGx Imp	Function	0/1	1/1
ABCA1	chr11	60809270	TGA	T	Extended	Transporter	1	0
ADH4	chr32	21295994	GTTTTTT	GTTTTT	Extended	Phase-I	3	0
ALDH3B1	chr18	49807377	CTG	C	Extended	Phase-I	1	1
ALDH3B1	chr18	49807371	CT	C	Extended	Phase-I	0	1
ARVCF	chr26	29530257	TGGGGGG	GGGGTGGGGAGGGGGG	Related	Target/Receptor	0	1
CAR	chr38	21255656	GGTACGT	GGT	Extended	Modifier	6	1
CEBPB	chr24	36633990	CCCCCGGCGGGCCCCGGC	CCCCCGGC	Related	Unknown	1	0
CYP1A2	chr30	37820110	CAA	CA	Core	Phase-I	3	0
CYP1A2	chr30	37824002	CCCCCATCTAT	CCCCCATCTATCCCCATCTAT	Core	Phase-I	4	0
CYP2S1	chr1	112747078	G	GGC	Extended	Phase-I	1	0
CYP4A39	chr15	13668765	TCCC	TCC	Extended	Unknown	7	1
DLA-DQA	chr12	2224871	CTGT	CT	Related	Unknown	4	0
DLA-DRB1	chr12	2157296	A	AT	Related	Unknown	3	0
DLA-DRB1	chr12	2157345	GGT	GCCACGT,G	Related	Unknown	2	1
DLA-DRB1	chr12	2157297	A	AG	Related	Unknown	1	0
FLT4	chr11	1171662	GT	G	Related	Unknown	0	1
FLT4	chr11	1171641	GGGGCGGGCGGG	GGGGCGGG	Related	Unknown	1	0
METAP1	chr32	21208568	CTTTT	CTT	Extended	Phase-I	12	1
NFKB1	chr32	23948573	TTGTTCTGTT	TTGTT	Related	Unknown	1	0
PML	chr30	37215571	C	CA	Related	Unknown	7	1
SLC22A10	chr18	53608677	GCT	GT	Extended	Transporter	8	3
SOD1	chr31	26539786	CTATAT	CTAT	-	Modifier	9	0
TNFRSF8	chr2	84202155	GCAC	GCACAC	Related	Unknown	1	0
UGT2B31	chr13	58949074	C	CA	Extended	Phase-II	2	0

The allele frequencies of these pathogenic variants in the 24 samples studied was calculated as per Hardy Weinberg Equilibrium. The plot in figure 22, depicts the distribution of the mutations and their zygosity. The detailed information is available in table 15.

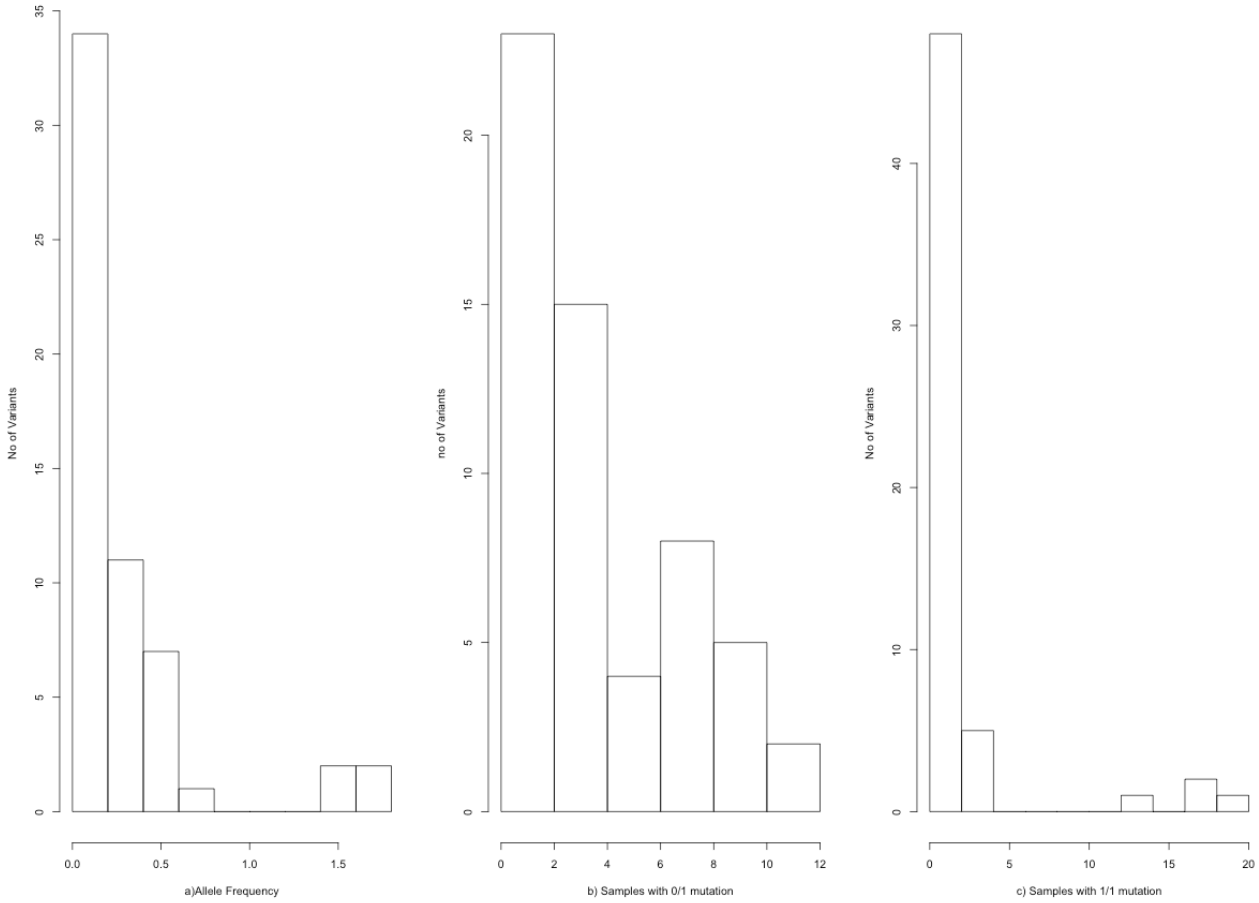


Figure 24. *Histograms for pathogenic variants with high confidence in the 24 Border collies analyzed representing the frequency of a) allele frequencies b) the number of samples that are carriers for the mutation c) the number of samples that are homozygous for the mutation.*

5.7 Known and Unknown Mutations

The pathogenic variants with high confidence were accessed across known clinical databases to verify if any of these were clinically relevant. The only online gene-variant database for dogs is Online Mendelian Inheritance for Animals (OMIA). To be able to use the information from human databases, the genomic co-ordinates of certainly pathogenic variants were converted to its orthologous base in human genome using liftOver. Based on the orthologous genomic positions of the canine variants, the human databases CliniVar, OMIM and PharmGKB were accessed for comparison (Figure 23). We found several examples of known pathogenic change in the dog genes when compared to human PGx gene variants. In this

process a few interesting phenotypes reported in and around the same region were identified. These studies were related to Cytochrome P-450 and the ABC transporters families.

CYP1A2, a member of the Cytochrome P 450 enzymes family is a PGx Core gene and a Phase-I metabolizing enzyme. The mutation identified as a part of this study, c.1117C>T, has an entry in OMIA and has been has identified to cause a truncated protein, when in a homozygous state, resulting in phenotypes of poor or extensive metabolizers in beagle dogs. In the current study , this mutation is not homozygous, but 7 out of 24 dogs pose to be carriers for this mutation (Mise, Hashizume, Matsumoto, Terauchi, & Fujii, 2004). Given the importance of CYP1A2 in the pharmacogenomics, this gene should be screened in additional Border Collies and other breeds to understand prevalence and to develop strategies to avoid adverse drug responses in future. CYP2J2 is another PGx Core, Phase –I enzyme, that has a mutation p.A407T in 2 Border Collies in heterozygous state. In humans a mutation p.N404T in the same gene has been identified to show significantly reduced metabolism of both arachidonic acid and linoleic acid in their homozygous state(King et al., 2002). It would be interesting to screen through other canine WGS data avialble for this mutation and check for possible phenotype and penetrance.

Among ABC transporters, three genes ABCA1, ABCB1 (MDR1) ,ABCC8 and ABCA1 had some interesting phenotypes in human. A missense mutation p.Q197H in MDR1 was identified in this study. In humans, a missense mutation p.G191R in the same protein domain has been identified and demonstrated to be resistant to many drugs in a set of leukemia patients, causing an efflux on the drug transport (Yang, Wu, Bui, & Ho, 2008). Therefore, it is possible that Q197H has clinical implications and should be further studied. MDR1(Multi Drug Resistant) is a highly sensitive gene towards many drugs both in human and canine. The well- known MDR1-delta(Δ) mutation, a four base pair deletion, in canine was not observed in this set of Border collies and has been found rare also in other studies (Katrina L Mealey, 2013). There was also another predicted pathogenic missense mutation p.S1485G in ABCC8 that requires further attention. The ABCC8 is a member of the ATP Binding Cassette, transporter family. There is a reported mutation in humans in the same protein nucleotide binding domain 2 (p.R1486K) that is a potential pathogenic mutation in case of patients with hyperinsulinemic hypoglycemia (HHG), a glucose metabolism disorder (Ohkubo et al., 2005). Another mutation R1420C, in the same nucleotide binding domain has been identified to be the cause the same disorder in infants (Tanizawa et al., 2000). Also it was observed

from mutdb that any mutation in this domain is highly pathogenic and is related to HHG. In a similar manner the frame shift mutation in ABCA1 (PGx extended and transporter) at p.I570 occurs in a protein domain that is highly sensitive to high density lipo-protein deficiency (HDLD) in humans. Mutations at this protein domain halt the release of lipids and cholesterol from cells causing hindrance to the production of HDL leading to HDLD.

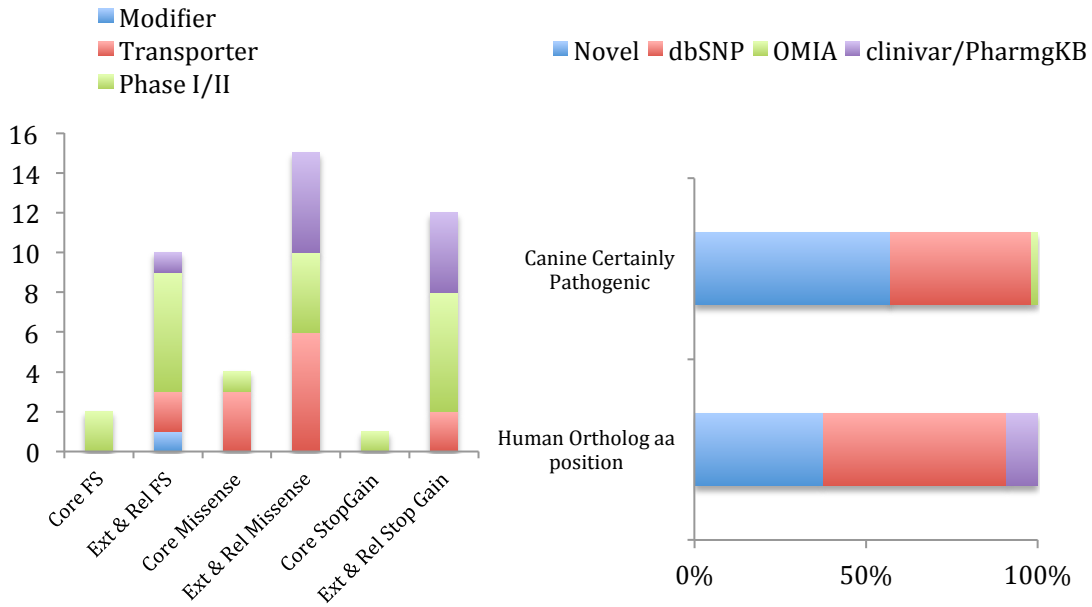


Figure 25. *Pathogenic indels and missense variants further classified based on their role in Pharmacogenomics*

5.8 DAVID Functional Enrichment analysis

To understand the biological significance behind the variants, functional annotation was performed for these genes using DAVID. The functional annotation was carried out for the pathogenic variants of high confidence to look for enriched pathways or protein domains. The enriched terms with a Benjamini Hochberg (BJ) adjusted pValue threshold less than 0.01 were selected. The details of the enriched pathways and protein domains are listed below in Table 15.

Table 15. The enriched pathway or mechanisms related to the pathogenic variants of high confidence and the genes involved in respective pathways or mechanisms in pharmacogenetics.

Category	Enriched Terms	Genes Involved	pValue	Adjusted (BJ) pValue
KEGG_PATHWAY	ABC transporters	ABCA1,ABCB4,ABCC10,ABCC11,ABCC8	1.4E-5	1.3E-3
KEGG_PATHWAY	Metabolism of xenobiotics by cytochrome P450	UGT2B31,ADH4,CYP1A2,CYP2S1,EPHX1	2.5E-5	1.2E-3

KEGG_PATHWAY	Drug metabolism - cytochrome P450	UGT2B31,ADH4,CYP1A2,FMO3	5.7E-4	1.9E-2
KEGG_PATHWAY	Retinol metabolism carcinogenesis	UGT2B31,ADH4,CYP1A2,CYP2S1	6.5E-4	1.6E-2

6. Discussion

Although the pharmacogenomic study in humans is quite well annotated and being carried out at increasing pace, it is still in an early developmental stage in dogs. This project aimed to build a bioinformatic pipeline for NGS data to identify and classify genetic variation in canine PGx genes in 24 Border Collies. The orthologues of the ~500 known human PGx genes were identified and screened for variations in the ORFs. Thousands of variants were identified and bioinformatic predictions indicate that about 1.3% of these could be harmful with expected phenotypes at least in the homozygous dogs. These likely pathogenic variants should be prioritized for experimental functional and clinical validation and for better estimation of the prevalence in the larger study cohorts within the Border collie and other breeds.

This pilot study establishes not only a useful bioinformatic tool and infrastructure for future studies but also reveals novel insights into the extent of the likely pathogenic PGx gene variation in dogs with implication to veterinary medicine. NGS data is rapidly accumulating in dogs and the approach developed here should greatly facilitate the identification of candidate variants for subsequent genetic, functional and clinical validations. The pipeline has been carefully designed by analyzing the results at the end of each phase that includes, ortholog identification, dna sequencing, variant identification and pathogen prediction, to ensure precise identification of the desired variants.

Many tools such as eggnog, UCSC Lift over, ensembl, and orthoMCL were reviewed to identify the canine orthologues. However, according to a few studies, the ensembl compara api has been evaluated to give best results for vertebrates when compared with other tools (Altenhoff & Dessimoz, 2009). Although the pseudo-genes identified by the compara API were not used in the further analysis in this study, they are saved in the in-house database for possible analyses. Using the ensembl api, 72% of the 27000 human genes have orthologues in canine genome. The percentage of genes with no orthologues can be related to the excess predicted genes and their paralog genes in human possibly due to spurious gene prediction (Lindblad-Toh et al., 2005). Contradictory to this, the human PGx list used in this project has about 90 % of orthologues in canine. This can be correlated with the study of parallel evolution between human and dogs (Wang et al., 2013), which states that most of the positively selected genes between human and canine belong to metabolism and digestion.

The pValue from the Chi-square test (0.001) provides additional support to this statement. From the eValues retrieved for the orthologue peptides, the highest value is '9e-21' (for CEBPD -an enhancer binding protein), which could still be considered a good eValue. About 75.02 % of the PGx orthologues have an eValue rounded to zero, as can be seen in Figure 15, suggesting that this percent of genes almost have no chance of the alignment occurring just by chance. Also from the histogram in Figure 15, depicting the ratio of base pairs in canine and human orthologue genes, it can be inferred that most of the orthologues have almost same length of open reading frame. The eValue and the gene length ratio provide additional confidence to use these human PGx genes as a reference to study canine pharmacogenomics. It is also interesting to note that genes with one to one orthologue relationship do not have any psuedogenes. The characterization of psuedogenes is very important, as it is evident from UGT1A1, a transferase enzyme producing gene, which is also a core PGx gene (Barbarino, Haidar, Klein, & Altman, n.d.). The human UGT1A1 has only a pseudogene as a canine orthologue, but the study by Troberg et al., provides details into the UGTs of canine and its pharmacogenomic relevance. (Troberg et al., 2014). More such studies are essential to understand the functionalities of the missing orthologues or psuedogenes. There has also been growing evidence that pseudogenes could be therapeutic targets. This information could be used to study how the small RNAs produced by pseudogene transcripts are involved with the regulation of gene expression of their genes (Roberts & Morris, 2013).

The high quality WGS data utilized here was generated by Illumina HiSeq2500 protocols. The steps followed in the sequencing pipeline are with reference to the GATK Best practices pipeline, which best suits to Illumina data. A good 99.3 percent of the reads were mapped after removing the low quality reads, representing a good sequencing library (Illumina Hi Seq) and alignment (BWA – MEM) combination. Although the percentage of unmapped reads is small (only 0.6%), and should not affect the storage of further performance much, they are still removed as well as the 3.39% of duplicate reads to avoid spurious variant calls. Samtools and GATK were utilized to identify variants in the WGS data. Although both tools are quite efficient in identifying variants, the combination of the tools helped to identify an additional 4% of the variants . Use of the combination of the tools has previously proven beneficial especially with the canine genome (Ahonen, Arumilli, & Lohi, 2013). We observed an overlap of 96% for SNPs but only 65% for indels between the tools. The haplotype caller is used to identify the variants with GATK and re-aligning the indels included in the pipeline ensures the confidence of the accuracy of the indels identified. Although the overall results

give an idea that the performance of GATK and Samtools is similar, the 4% difference in the SNPs can be related to the difference in the Bayesian genotyping method. Also the error modeling during GATK likelihood calculation, could cause the difference in the genotype calling from Samtools, especially when the depth is more than hundred folds. The 'genotypeMerge' option has been used to take care of the variants with genotype differences between samtools and GATK, by prioritizing the GATK genotypes. This suggests that use of combination of tools or algorithms is always a better choice to increase the accuracy and rate of novel variants identified. The same procedure could also be used with exome sequencing samples, as this project only intends to look at the open reading frames of the genes. The NGS pipeline used has been developed in such a way that, the variant files are made available in a standard VCF 4.0 format, so that they could be used for any other research project if required.

Filtering the variants based on the open reading frame positions was the initial step to use VCFs from the whole genome samples. This step significantly reduced the storage space as the interested regions (ORFs of the PGx genes), comprised only about 0.1% of the whole genome variant file. This change would be quite radical even for an exome variant file, as the PGx ORFs contribute to only about 1% of the whole canine exome region. This decreases the computational space and time required for further processing.

SnpEff has been a very useful tool to predict the effect of the variants on the coding sequences. Based on the type of variation, an impact factor is assigned to it. The impact factor has been quite useful to further filter the variants. Other than missense SNPs, that are classified to have moderate impact by SnpEff, high impact variations were selected to proceed further. Among the high impact variations there were only frame-shift, stop gain or stop lost mutations. As the project looks at only the open reading frames, it is possible than only these types of variations occurred. Also information such as name of the gene, change in the amino acid sequence due to the variation, exon number, name of the transcript, etc., were also annotated using SnpEff. To be able to differentiate between novel and known variations, the variants were annotated with variants from canine dbSNP. In this whole process Ensembl database was used, as the same is being followed throughout, from identifying the orthologues to predicting the pathogenic variants.

As not all missense variants are pathogenic, they were run using two separate pathogenic tools, Polyphen2 and SIFT. Although the total number of variants predicted deleterious with a

good confidence were quite close, 200 by Polyphen2 and 230 by SIFT, the number of variants identified commonly by both the tools is only 27, which is only about 7% to 8% of the individual pathogenic sets. The reason for this is that, both the tools classify them into different categories of pathogenicity. Polyphen2 classifies them into possibly damaging, probably damaging and neutral with a Bayesian score. SIFT classifies into predicted deleterious, tolerated and neutral with different confidence type, high confidence and low confidence. As only a strict selection criterion was used, the missense variations that were predicted as absolutely deleterious were selected. This selection criterion suits the PGx study as mentioned in bioinformatics approach to achieve translation medicine from NGS data (Figure 3 in the Introduction section). There are also other studies that support the approach of using a combination of multiple pathogen prediction tools to ensure the confidence of pathogenicity(Grimm et al., 2015)(Hicks, Wheeler, Plon, & Kimmel, 2011). Although there is a debate on reliability on pathogen prediction tools, due to possibility of missing true positives, it is still an efficient method to prioritize the variants for functional validation.

The key challenge in the whole process was validation of the indels using IGV, as the task demanded more manual work. The accurate detection of indels is still a challenge due to reasons such as low coverage and presence of repeated sequences. The study by Yue Jiang et al suggests that the indels reported by different studies on a same population are highly inconsistent(Jiang, Turinsky, & Brudno, 2015). Such studies also support the necessity of more accurate indel detection tool and high throughput data with better quality. The other interesting set of variants was the presence of homozygous variants in all the samples. These variants could be either specific to the reference genome Boxer or the Border Collies. Analyzing the same variants in exomes or whole genomes of other breeds could give a better insight. However as this scenario has appeared only in indels (frame shift) it would be appropriate to re-call the indels using different parameters before drawing conclusions. These facts suggest that there is still a lot of scope for better indel calling algorithms.

The certainly pathogenic variants contribute to about 6% of variant set studied for pathogenicity. Figure 23 (in the Results section) clearly depicts the portion of likely, unlikely and pathogenic with high confidence variants. While the small portion of final variants depicts the confidence in the pathogenic variants, the 25% of likely pathogenic variants (low-confidence) is the result of difference in pathogen prediction algorithms. This also depicts the

need for a universal prediction algorithm that can detect pathogenic variants with more accuracy.

Recent studies have suggested that many genetic variants that are pathogenic are common in asymptomatic individuals (Cassa, Tong, & Jordan, 2013). The study of functional variants in human PGx genes by Nelson R.et al, depicts that there is an abundance in the number of deleterious rare variants that have a functional impact on drug metabolism. This pattern of distribution highlights the necessity to analyze the allele frequency of PGx mutations in a population (Nelson et al., 2012). In most of the cases the adverse reaction to drugs is caused by homozygous mutations. In addition to being carriers, heterozygous mutations can sometimes cause adverse reaction with reduced intensity. For example in the analysis of human CYP genes van der weide et al, categorized the mutations into poor, intermediate and extensive metabolizers based on the genotype of the mutations (van der Weide & Hinrichs, 2006). Hence understanding of the distribution of allele frequencies and zygosity is quite essential. From the figure 22.a, it can be seen that in the Border collie population studied a huge proportion of the pathogenic variants are rare and specific to samples. It can also be inferred from the figure 22.b,c that the proportion of the heterozygote mutations are more when compared to homozygous mutations. The outlier in the histogram represent variants from DLA-DQA1 and DLA-DRB1 which is are highly polymorphic genes (Kennedy et al., 2000). From the David functional enrichment analysis, it can be seen that the enriched pathways are transporters and metabolism related. Previous interest has also targeted transporters (Katrina L Mealey, 2013) and metabolizing enzymes (Court, 2013). As the canine genome has possibilities for new assembly and annotation in future, updating the annotation table and re-running that last step of the table is sufficient to keep the analyzed and new data updated. Also the different phases of the pipeline are independent of the other and hence can be upgraded or optimized easily whenever there is possibility.

7. Conclusion and future prospects

The increase in the need to understand personalized medicine, due to ineffective or toxic responses of a group of individuals to certain drugs, has led to tremendous increase in the study of pharmacogenomics in the past four to five years. This project aimed at accomplishing the first two stages of the 'bioinformatics approach to achieve personalized medicine' that are, processing of large-scale genomic data and functional interpretation of the effect and impact of the genomic variation. Identification of the pathogenic variants can be further validated and analyzed for possibility of being a potential biomarker. The output could be further integrated with other biological data, such as proteomic and metabolomics data, using systems biology approaches to achieve translational or personalized medicine.

This study had practical and scientific implications. We established a powerful bioinformatic infrastructure that allows systematic study of NGS data for PGx variation in future. A precise set of PGx genes was gathered and respective canine orthologs were identified. After detailed analysis, the canine PGx set was included in the analysis. The PGx gene set was also annotated with labels, based on the gene's role and importance in drug processing. A standard pipeline was established using the state of the art techniques to perform the NGS analysis. The pipeline was designed in such a way, that it could process, either WGS or WES data. The final output of the data included a set of pathogenic variants that were predicted to have pharmacogenomic implications in the population studied. The framework also produced a report on the frequency, pathway analysis and allele zygosity of the pathogenic variants. The pipeline is designed in an object or module oriented design. As a result, each module is independent of each other and any future changes for optimization would be easy and would not affect the other modules.

This mode of analysis could be highly useful to study the clinical impact, since we already discovered several putative functional variants, in the small pilot of 24 samples. Ongoing analyses in about 500 samples, following a genotype to phenotype pattern of research, should reveal many new candidate variants, especially in the core PGx genes. It would also establish a large study of functional and clinical pharmacogenomics in dogs, contributing to translational and personalized medicine.

8. References

Adzhubei, I., Jordan, D. M., & Sunyaev, S. R. (2013). Predicting functional effect of human missense mutations using PolyPhen-2. *Current Protocols in Human Genetics*. doi:10.1002/0471142905.hg0720s76

Ahonen, S. J., Arumilli, M., & Lohi, H. (2013). A CNGB1 Frameshift Mutation in Papillon and Phalène Dogs with Progressive Retinal Atrophy. *PLoS ONE*, *8*. doi:10.1371/journal.pone.0072122

Altenhoff, A. M., & Dessimoz, C. (2009). Phylogenetic and functional assessment of orthologs inference projects and methods. *PLoS Computational Biology*, *5*. doi:10.1371/journal.pcbi.1000262

Bai, B., Zhao, W. M., Tang, B. X., Wang, Y. Q., Wang, L., Zhang, Z., … Zhang, Y. P. (2015). DoGSD: The dog and Wolf genome SNP database. *Nucleic Acids Research*, *43*, D777–D783. doi:10.1093/nar/gku1174

Barbarino, J. M., Haidar, C. E., Klein, T. E., & Altman, R. B. (n.d.). PharmGKB summary: very important pharmacogene information for UGT1A1. doi:10.1097/FPC.0000000000000024

Blankenberg, D., Gordon, A., Von Kuster, G., Coraor, N., Taylor, J., Nekrutenko, A., & Team, G. (2010). Manipulation of FASTQ data with galaxy. *Bioinformatics*, *26*, 1783–1785. doi:10.1093/bioinformatics/btq281

Cassa, C. A., Tong, M. Y., & Jordan, D. M. (2013). Large numbers of genetic variants considered to be pathogenic are common in asymptomatic individuals. *Human Mutation*, *34*, 1216–1220. doi:10.1002/humu.22375

Chan, I. S., & Ginsburg, G. S. (2011). Personalized medicine: progress and promise. *Annu Rev Genomics Hum Genet*, *12*, 217–44. doi:10.1146/annurev-genom-082410-101446

Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., … Ruden, D. M. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w 1118; iso-2; iso-3. *Fly*, *6*, 80–92. doi:10.4161/fly.19695

Cordero, P., & Ashley, E. a. (2012). Whole-genome sequencing in personalized therapeutics. *Clinical Pharmacology and Therapeutics*, *91*, 1001–9. doi:10.1038/clpt.2012.51

Court, M. H. (2013). Canine Cytochrome P-450 Pharmacogenetics. *Veterinary Clinics of North America - Small Animal Practice*. doi:10.1016/j.cvsm.2013.05.001

DePristo, M. A., Banks, E., Poplin, R., Garimella, K. V, Maguire, J. R., Hartl, C., … Daly, M. J. (2011). A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature Genetics*, *43*, 491–8. doi:10.1038/ng.806

Doolittle, W. F. (1999). Phylogenetic classification and the universal tree. *Science (New York, N.Y.)*, *284*, 2124–2129. doi:10.1126/science.284.5423.2124

Ebbert, M. T. W., Wadsworth, M. E., Staley, L. A., Hoyt, K. L., Pickett, B., Miller, J., … Shyr, Y. (2016). Evaluating the necessity of PCR duplicate removal from next-generation sequencing data and a comparison of approaches. *BMC Bioinformatics*, *17*, 239. doi:10.1186/s12859-016-1097-3

Fernald, G. H., Capriotti, E., Daneshjou, R., Karczewski, K. J., & Altman, R. B. (2011a). Bioinformatics challenges for personalized medicine. *Bioinformatics*. doi:10.1093/bioinformatics/btr295

Fernald, G. H., Capriotti, E., Daneshjou, R., Karczewski, K. J., & Altman, R. B. (2011b). Bioinformatics challenges for personalized medicine. *Bioinformatics*, *27*, 1741–1748. doi:10.1093/bioinformatics/btr295

Fleischer, S., Sharkey, M., Mealey, K., Ostrander, E. A., & Martinez, M. (2008). Pharmacogenetic and metabolic differences between dog breeds: Their impact on canine medicine and the use of the dog as a preclinical animal model. *AAPS Journal*, *10*, 110–119. doi:10.1208/s12248-008-9011-1

Frank, R., & Hargreaves, R. (2003). Clinical biomarkers in drug discovery and development. *Nature Reviews. Drug Discovery*, *2*, 566–80. doi:10.1038/nrd1130

Gaedigk, A., Sangkuhl, K., Whirl-Carrillo, M., Klein, T., & Leeder, J. S. (2016). Prediction of CYP2D6 phenotype from genotype across world populations. *Genetics in Medicine : Official Journal of the American College of Medical Genetics*. doi:10.1038/gim.2016.80

Garc??a-Alcalde, F., Okonechnikov, K., Carbonell, J., Cruz, L. M., G??tz, S., Tarazona, S., … Conesa, A. (2012). Qualimap: Evaluating next-generation sequencing alignment data. *Bioinformatics*, *28*, 2678–2679. doi:10.1093/bioinformatics/bts503

Ginsburg, G. S., & Willard, H. F. (2009). Genomic and personalized medicine: foundations and applications. *Translational Research*. doi:10.1016/j.trsl.2009.09.005

Gouin, a., Legeai, F., Nouhaud, P., Whibley, a., Simon, J.-C., & Lemaitre, C. (2014). Whole-genome re-sequencing of non-model organisms: lessons from unmapped reads. *Heredity*, *114*, 494–501. doi:10.1038/hdy.2014.85

Grimm, D. G., Azencott, C.-A., Aicheler, F., Gieraths, U., MacArthur, D. G., Samocha, K. E., … Borgwardt, K. M. (2015). The evaluation of tools used to predict the impact of missense variants is hindered by two types of circularity. *Human Mutation*, *36*(5), 513–23. doi:10.1002/humu.22768

Haller, S., Schuler, F., Lazic, S. E., Bachir-Cherif, D., Krämer, S. D., Parrott, N. J., … Belli, S. (2012). Expression Profiles of Metabolic Enzymes and Drug Transporters in the Liver and along the Intestine of Beagle Dogs. *Drug Metabolism and Disposition*, *40*, 1603–1611. doi:10.1124/dmd.112.045443

Hicks, S., Wheeler, D. A., Plon, S. E., & Kimmel, M. (2011). Prediction of missense mutation functionality depends on both the algorithm and sequence alignment employed. *Human Mutation*, *32*, 661–668. doi:10.1002/humu.21490

International Warfarin Pharmacogenetics Consortium, Klein, T. E., Altman, R. B., Eriksson, N., Gage, B. F., Kimmel, S. E., … Johnson, J. A. (2009). Estimation of the warfarin dose with clinical and pharmacogenetic data. *The New England Journal of Medicine*, *360*, 753–64. doi:10.1056/NEJMoa0809329

Jiang, Y., Turinsky, A. L., & Brudno, M. (2015). The missing indels: An estimate of indel variation in a human genome and analysis of factors that impede detection. *Nucleic Acids Research*, *43*, 7217–7228. doi:10.1093/nar/gkv677

Johnson, J. A. (2003). Pharmacogenetics: Potential for individualized drug therapy through genetics. *Trends in Genetics*. doi:10.1016/j.tig.2003.09.008

Kennedy, L. J., Altet, L., Angles, J. M., Barnes, A., Carter, S. D., Francino, O., … Wagner, J. L. (2000). Nomenclature for factors of the Dog Major Histocompatibility System (DLA), 1998: First report of the ISAG DLA Nomenclature Committee. *Animal Genetics*, *31*, 52–61. doi:10.1046/j.1365-2052.2000.00492.x

King, L. M., Ma, J., Srettabunjong, S., Graves, J., Bradbury, J. A., Li, L., … Zeldin, D. C. (2002). Cloning of CYP2J2 gene and identification of functional polymorphisms. *Molecular Pharmacology*, *61*, 840–52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11901223

Kwon, S., Park, S., Lee, B., & Yoon, S. (2013). In-depth analysis of interrelation between quality scores and real errors in Illumina reads. In *Proceedings of the Annual International Conference of the IEEE Engineering in Medicine and Biology Society, EMBS* (pp. 635–638). doi:10.1109/EMBC.2013.6609580

Li, H. (2011). A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics*, *27*, 2987–2993. doi:10.1093/bioinformatics/btr509

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., … Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*, 2078–2079. doi:10.1093/bioinformatics/btp352

Li, W., Yang, W., & Wang, X.-J. (2013). Pseudogenes: Pseudo or Real Functional Elements? *Journal of Genetics and Genomics*, *40*, 171–177. doi:10.1016/j.jgg.2013.03.003

Lindblad-Toh, K., Wade, C. M., Mikkelsen, T. S., Karlsson, E. K., Jaffe, D. B., Kamal, M., … Lander, E. S. (2005). Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature*, *438*, 803–819. doi:10.1038/nature04338

Mealey, K. L. (2006). Pharmacogenetics. *The Veterinary Clinics of North America. Small Animal Practice*, *36*, 961–73, v. doi:10.1016/j.cvsm.2006.05.006

Mealey, K. L. (2013). Adverse drug reactions in veterinary patients associated with drug transporters. *The Veterinary Clinics of North America. Small Animal Practice*, *43*, 1067–78. doi:10.1016/j.cvsm.2013.04.004

Mealey, K. L., Bentjen, S. a, Gay, J. M., & Cantor, G. H. (2001). Ivermectin sensitivity in collies is associated with a deletion mutation of the mdr1 gene. *Pharmacogenetics*, *11*, 727–733. doi:10.1097/00008571-200111000-00012

Mealey, K. L., Fidel, J., Gay, J. M., Impellizeri, J. A., Clifford, C. A., & Bergman, P. J. (2008). ABCB1-1?? polymorphism can predict hematologic toxicity in dogs treated with vincristine. *Journal of Veterinary Internal Medicine*, *22*, 996–1000. doi:10.1111/j.1939-1676.2008.0122.x

Messing, J., & Llaca, V. (1998). Importance of anchor genomes for any plant genome project. *Proceedings of the National Academy of Sciences of the United States of America*, *95*, 2017–2020. doi:10.1073/pnas.95.5.2017

Mise, M., Hashizume, T., Matsumoto, S., Terauchi, Y., & Fujii, T. (2004). Identification of non-functional allelic variant of CYP1A2 in dogs. *Pharmacogenetics*, *14*, 769–773. doi:10.1097/00008571-200411000-00008

Mosher, C. M., & Court, M. H. (2010). Comparative and veterinary pharmacogenomics. *Handbook of Experimental Pharmacology*, *199*, 49–77. doi:10.1007/978-3-642-10324-7

Nelson, M. R., Wegmann, D., Ehm, M. G., Kessner, D., St. Jean, P., Verzilli, C., … Mooser, V. (2012). An Abundance of Rare Functional Variants in 202 Drug Target Genes Sequenced in 14,002 People. *Science*, *337*, 100–104. doi:10.1126/science.1217876

Ng, P. C., & Henikoff, S. (2001). Predicting deleterious amino acid substitutions. *Genome Research*, *11*, 863–874. doi:10.1101/gr.176601

Ng, P. C., & Henikoff, S. (2006). Predicting the effects of amino acid substitutions on protein function. *Annual Review of Genomics and Human Genetics*, *7*, 61–80. doi:10.1146/annurev.genom.7.080505.115630

Nielsen, R., Paul, J. S., Albrechtsen, A., & Song, Y. S. (2011). Genotype and SNP calling from next-generation sequencing data. *Nature Reviews. Genetics*, *12*, 443–451. doi:10.1038/nrg2986

Ohkubo, K., Nagashima, M., Naito, Y., Taguchi, T., Suita, S., Okamoto, N., … Ono, J. (2005). Genotypes of the pancreatic beta-cell K-ATP channel and clinical phenotypes of Japanese patients with persistent hyperinsulinaemic hypoglycaemia of infancy. *Clinical Endocrinology*, *62*, 458–65. doi:10.1111/j.1365-2265.2005.02242.x

Ozawa, N., Shimizu, T., Morita, R., Yokono, Y., Ochiai, T., Munesada, K., … Sugiyama, Y. (2004). Transporter database, TP-search: A web-accessible comprehensive database for research in pharmacokinetics of drugs. *Pharmaceutical Research*. doi:10.1023/B:PHAM.0000048207.11160.d0

Ranieri, G., Gadaleta, C. D., Patruno, R., Zizzo, N., Daidone, M. G., Hansson, M. G., … Ribatti, D. (2013). A model of study for human cancer: Spontaneous occurring tumors in dogs. Biological features and translation for new anticancer therapies. *Critical Reviews in Oncology/Hematology*. doi:10.1016/j.critrevonc.2013.03.005

Relling, M. (2015). Clinical implementation of pharmacogenetics: CPIC guidelines. *Clinical Chemistry and Laboratory Medicine*, *53*, S75. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed13&NEWS=N&AN=71910363

Roberts, T. C., & Morris, K. V. (2013). Not so pseudo anymore: pseudogenes as therapeutic targets. *Pharmacogenomics*, *14*, 2023–34. doi:10.2217/pgs.13.172

Rodriguez-Antona, C. (2015). The role of pharmacogenetics and pharmacogenomics in 21st-century medicine: state of the art and new challenges discussed in the VII Conference of the Spanish. *Drug Metabolism and Personalized Therapy*, 15–16. doi:10.1515/dmpt-2015-0033

Salavaggione, O., & Kidd, L. (2002). Canine red blood cell thiopurine S-methyltransferase: companion animal pharmacogenetics. *Pharmacogenetics*, *12*, 713–724. doi:10.1124/jpet.103.059055.provide

Seppälä, E. H., Jokinen, T. S., Fukata, M., Fukata, Y., Webster, M. T., Karlsson, E. K., … Lohi, H. (2011). Lgi2 truncation causes a remitting focal epilepsy in dogs. *PLoS Genetics*, *7*. doi:10.1371/journal.pgen.1002194

Shendure, J., & Ji, H. (2008). Next-generation DNA sequencing. *Nat Biotechnol*, *26*, 1135–1145. doi:10.1038/nbt1486

Shin, J., Kayser, S. R., & Langaee, T. Y. (2009). Pharmacogenetics: From discovery to patient care. *American Journal of Health-System Pharmacy*. doi:10.2146/ajhp080170

Sim, E., Fakis, G., Laurieri, N., & Boukouvala, S. (2012). Arylamine N-acetyltransferases--from drug metabolism and pharmacogenetics to identification of novel targets for pharmacological intervention. *Adv Pharmacol*, *63*, 169–205. doi:10.1016/B978-0-12-398339-8.00005-7

Sim, S. C., Altman, R. B., & Ingelman-Sundberg, M. (2011). Databases in the area of pharmacogenetics. *Human Mutation*. doi:10.1002/humu.21454

Sim, S. C., & Ingelman-Sundberg, M. (2013). Update on allele nomenclature for human cytochromes P450 and the Human Cytochrome P450 Allele (CYP-allele) Nomenclature Database. *Methods in Molecular Biology (Clifton, N.J.)*, *987*, 251–259. doi:10.1007/978-1-62703-321-3_21

Sims, D., Sudbery, I., Ilott, N. E., Heger, A., & Ponting, C. P. (2014). Sequencing depth and coverage: key considerations in genomic analyses. *Nature Reviews Genetics*, *15*, 121–32. doi:10.1038/nrg3642

Studer, R. A., & Robinson-Rechavi, M. (2009). How confident can we be that orthologs are similar, but paralogs differ? *Trends in Genetics*, *25*, 210–216. doi:10.1016/j.tig.2009.03.004

Tanizawa, Y., Matsuda, K., Matsuo, M., Ohta, Y., Ochi, N., Adachi, M., … Oka, Y. (2000). Genetic analysis of Japanese patients with persistent hyperinsulinemic hypoglycemia of

infancy: Nucleotide-binding fold-2 mutation impairs cooperative binding of adenine nucleotides to sulfonylurea receptor 1. *Diabetes*, *49*, 114–120.

The Cost of Sequencing a Human Genome. (n.d.). Retrieved from https://www.genome.gov/27565109/the-cost-of-sequencing-a-human-genome/

Thorn, C. F., Klein, T. E., & Altman, R. B. (2005). PharmGKB: the pharmacogenetics and pharmacogenomics knowledge base. In *Methods in molecular biology* (Vol. 311, pp. 179–191). doi:10.1038/sj.tpj.6500230

Thorn, C. F., Klein, T. E., & Altman, R. B. (2010). Pharmacogenomics and bioinformatics: PharmGKB. *Pharmacogenomics*, *11*, 501–505. doi:10.2217/pgs.10.15

Tin, M. M. Y., Rheindt, F. E., Cros, E., & Mikheyev, A. S. (2014). Degenerate adaptor sequences for detecting PCR duplicates in reduced representation sequencing data improve genotype calling accuracy. *Molecular Ecology Resources*, *15*, 329–36. doi:10.1111/1755-0998.12314

Troberg, J., Jarvinen, E., Muniz, M., Sneitz, N., Mosorin, J., Hagstrom, M., & Finel, M. (2014). The Dog UGT Enzymes of Subfamily 1a; Cloning, Expression and Activity. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, dmd.114.059303–. doi:10.1124/dmd.114.059303

Van der Weide, J., & Hinrichs, J. W. J. (2006). The influence of cytochrome P450 pharmacogenetics on disposition of common antidepressant and antipsychotic medications. *The Clinical Biochemist. Reviews / Australian Association of Clinical Biochemists*, *27*, 17–25. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16886044\nhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1390790

Vilella, A. J., Severin, J., Ureta-Vidal, A., Heng, L., Durbin, R., & Birney, E. (2009). EnsemblCompara GeneTrees: Complete, duplication-aware phylogenetic trees in vertebrates. *Genome Research*, *19*, 327–335. doi:10.1101/gr.073585.107

Wang, G. D., Zhai, W., Yang, H. C., Fan, R. X., Cao, X., Zhong, L., … Zhang, Y. P. (2013). The genomics of selection in dogs and the parallel evolution between dogs and humans. *Nat Commun*, *4*, 1860. doi:10.1038/ncomms2814

Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P., … Lander, E. S. (2002). Initial sequencing and comparative analysis of the mouse genome. *Nature*, *420*, 520–562. doi:10.1038/nature01262\rnature01262 [pii]

Yang, Z., Wu, D., Bui, T., & Ho, R. J. Y. (2008). A novel human multidrug resistance gene MDR1 variant G571A (G191R) modulates cancer drug resistance and efflux transport. *The Journal of Pharmacology and Experimental Therapeutics*, *327*, 474–481. doi:jpet.108.138313 [pii]\r10.1124/jpet.108.138313

Yu, X., & Sun, S. (2013). Comparing a few SNP calling algorithms using low-coverage sequencing data. *BMC Bioinformatics*, *14*, 274. doi:10.1186/1471-2105-14-274

Zhou, Y., Gottesman, M., & Pastan, I. (1999). Studies of human MDR1-MDR2 chimeras demonstrate the functional exchangeability of a major transmembrane segment of the multidrug transporter and phosphatidylcholine flippase. *Molecular and Cellular Biology*, *19*, 1450–1459. Retrieved from /Users/Maria/Dropbox/Posgrado/pappers/Library/pdf0/2765.pdf

9. Appendix

9.1 File Formats

9.1.1 Fastq

The Fastq Files contain two types of lines. One has the read identifier that starts with the symbol '>' and the next succeeding line is the nucleotide (or amino acid if it is a protein fasta file) sequence of the particular read (or transcript for protein fasta). Once the sequence is completed the next line again starts with the identifier of the next sequence and it goes on so. As mentioned the sequence identifier starts '>' and is immediately followed by the name or id of the sequence. The id or name could be a combination of information. In the example below, it can be seen that the identifier starts with '>' and is followed by gene name, ensembl gene id, transcript id and protein name separated by ':'. The second line depicts the nucleotide sequence.

```
>ACBA1:ENSCAFG00000002727:ENSCAFT00000004346:ABCA1
TTAATGACCAGCCACGGCGGCCGCCTCCACGGGCCCCCGAGCCACACGCACGGTGCTGGCGCCGCCCGCTGAGCCGACATGG
CCTCCTGGCCTCAGCTGCGCCTGCTGCTGTGGAAGAACCTCACCTTCAGAAGAAGGCAGACCTGTCAGCTGTTGCTGGAAGTG
GCCTGGCCTCTGTTTATCTTCCTGATCCTGATCTCCGTGCGGCTGAGCTACCCGCCCTATGAGCAACATGAGTGCCACTTTCCA
AACAAAGCCATGCCCTCTGCAGGAACGCTTCCTTGGATTCAGGGGATTATCTGTAATGCCAATAACCCCTGCTTCCGCTATCC
GACTCCTGGTGAGGCCCCTGGGGTGGTTGGAAACTTTAACAAATCCATTGTGTCTCGCCTGTTCTCAGATGCTCAGCGGCTCC
TTTTA
```

9.1.2 BAM

Binary Alignment Map or BAM file is a binary form of sequence alignment data.

```
HISEQ:16:D24BTACXX:6:2301:1341:71647    163    chr1 112734887    60    101M    =    112735185
    399
    TCAGTCAGTGGAGCGTGCACTCTTGATCTCGGGGTGGGGTGGGGGTGTTTGAGTTTGAGCCCCACGTTGGGTGGAA
AGAGGACTTAGAAACAAAAGCTTAA
    =>:>===>=>===<4;<;<;<<<<<<<<<<4;;;/9;<<.69;;;6799<;::9:<=:<:;<<<<5:==<<:9;;<<;2:;<;=???>??>>??>==>==?
    MC:Z:101M
    BD:Z:EEHHKKJJIGIGDIEGFGHDGDDDFGFFDDCDBBEEGBBEEGBBBEEEE?FGCHF@GHDIFDDGEFGFGHCFFHGF
@EDDGHHIFEIEGBHHJCCIMHEDE    MD:Z:101
    BI:Z:FFHHHGHHGEGGEGEFEEHDGDECEGFEEDCEDDEEGDDEEGDDDEEFEAEGEFEBFHFHGEEHEGGFFHEFFHH
GBFGGHIFIEGIHIDHJKFFJMIDFF    NM:i:0    MQ:i:60    AS:i:101    XS:i:0    RG:Z:BC223-5B5E6E0A
```

9.1.3 VCF

Variant Call Format (VCF) is a tab-separated file that has details of the NGS variants. The VCF file was initially created by the 1000 genomes. It starts with a meta-information line, header and then followed by data. The meta-information lines starts with '##' and each line contains details of the file such as format or information of the header, such as, Chromosome (CHROM), position (POS), Reference (REF), etc. The fields of the VCF files are mentioned here below

CHROM	Chromosome Number
POS	Position of the variant
ID	Variant ID eg/- BICF2S2345117
REF	The reference nucleotide at the given chromosome position
ALT	The alternate nucleotide/variant in the sample at the same position
QUAL	Quality of the read at the chromosomal position
FILTER	Denotes if the position has passed all the filters
INFO	Denotes other information such as Allele frequency, allele count, depth, sample genotype etc. Also the annotation is included in this column.

9.1.4 Bed File

A bed file is a tab-delimited file with information about a genomic region. There are three mandatory regions in a bed file, chromosome name, region start and region end. There are nine other optional fields such as name, strand etc., and providing information about the genomic region in the mandatory field. The below is an example of a basic bed file used in this project.

```
>CYP1A2.bed
chr30   37819355        37820173
chr30   37820699        37820819
chr30   37821253        37821342
chr30   37821600        37821723
chr30   37822620        37822706
chr30   37823781        37824078
```

9.1.5 Polyphen 2 Input

The input is a tab-delimited text with protein name, position of the alteration, reference and alternate amino acids. The alternate amino acid is the substitution for the reference amino acid due to the variation. The protein identifier can be protein name, Uniprot or ensembl or Ref Seq name. In this case the ensembl protein identifier is retrieved from translation adaptor(ensembl perl api) using transcript id, which in turn can be obtained from vcf file using the SnpSift ‘extractFields’ utility in SnpEff tool.

Protein Identifier	Position in Sequence	Amino Acids Ref	Amino Acids Alt
ENSCAFP00000001840	22	R	P

9.2 Key Commands and Arguments

9.2.1 Retrieve Orthologs(Ensembl Compara Perl API)

```
my $gene_member_adaptor =$reg->get_adaptor( "Multi", "compara", "GeneMember" );
my $homology_adaptor = $reg->get_adaptor( "Multi", "compara", "Homology" );
my $gene_member =$gene_member_adaptor->fetch_by_source_stable_id( 'ENSEMBLGENE',$geneEId );
my $dog_ortholougues =$homology_adaptor->fetch_all_by_Member_paired_species($gene_member, "Canis lupus familiaris",['ENSEMBL_ORTHOLOGUES'] );
```

9.2.2 WGS

Pre-processing Reads

```
gunzip -c <fastq1> |<path_to_fastx>/fastq_quality_filter -i - -o <fastq1_filtered> -v -Q 33 -q 20 ;

gunzip -c <fastq2> |<path_to_fastx>/fastq_quality_filter -i - -o <fastq2_filtered> -v -Q 33 -q 20 ;
```

Align against Reference

```
bwa mem -R '@RG\tID:bwa\tLB:'<Sample_Name>'\tSM:'<SampleName>'\tPL:ILLUMINA' <canFam3.1> <fastq1_filtered>  <fastq2_filtered> | samtools view -bS - > <alignment_bam>
```

Pre-processing Aligned Reads

```
#Sort Bam file
samtools sort <alignment_bam> <alignment_sorted_bam>
#Remove unmapped reads
samtools_0.1.18 view -bF 4 <alignment_sorted_bam> > <alignment_sorted_MR>
#Mark duplicates
java -jar picard.jar MarkDuplicates  INPUT=<alignment_sorted_MR> OUTPUT=<alignment_sorted_MF_dedup>

#Calculate coverage
qualimap bamqc -bam <alignment_sorted_MF_dedup>  -outformat PDF  -c > <Alignment_Coverage_Stats>
```

Indel Re-alignment and Recalibration

```
#Identify intervals
java -jar GenomeAnalysisTK.jar -T RealignerTargetCreator -R <canFam3.1> -I <alignment_sorted_MF_dedup>  -o <alignment_realign.intervals>

#Realign around intervals
java -Xmx4g -jar GenomeAnalysisTK.jar -T IndelRealigner -R <canFam3.1>  -I <alignment_sorted_MF_dedup> -targetIntervals <alignment_realign.intervals> -o <alignment_realign_bam>

#Re-calibration
```

java -Xmx4g -jar GenomeAnalysisTK.jar -T BaseRecalibrator -R <canFam3.1> -knownSites <dogGSD.vcf> -I <alignment_realign_bam> -o <alignment_realign_recal>
java -Xmx4g -jarGenomeAnalysisTK.jar -T PrintReads -R <canFam3.1> -I <alignment_realign_bam> -BQSR <alignment_realign_recal> -o <alignment_recalibrated.bam>

Identify Variants with GATK

#GATK variants identification

java -jar GenomeAnalysisTK.jar -R <canFam3.1> -T HaplotypeCaller -I <alignment_recalibrated.bam> -stand_emit_conf 10 -stand_call_conf 30 --genotyping_mode DISCOVERY -o <GATK.vcf>

#Filter with depth and remove others

java -jar GenomeAnalysisTK.jar -R <canFam3.1> -T VariantFiltration --variant <GatkSNP_raw.vcf> --filterExpression 'QD < 2.0' --filterName QD --filterExpression 'MQ < 40.0' --filterName MQ --filterExpression 'DP < 10' --filterName DP -o <Gatk_filt.vcf>

java -jar GenomeAnalysisTK.jar -R <canFam3.1> -T SelectVariants --variant - --excludeFiltered -o <Gatkfilt_Sel.vcf>

Identify Variants with Samtools

samtools_0.1.18 mpileup -ABugf <canFam3.1> -d 1000000 <alignment_recalibrated.bam> | bcftools view -vcg - | bcfTools/vcfutils.pl varFilter -D 1000000 > <Sam.vcf>

##Filter SNPS

bcftools/vcfutils.pl varFilter -Q 40 -d 10 <Sam.vcf> | awk '$6>=40' > <Sam_filt.vcf>

Combine both GATK and Samtools

java -jar GenomeAnalysisTK.jar -T CombineVariants -R <canFam3.1> --variant:Gatk <Gatkfilt_Sel.vcf> --variant:Sam <Sam_filt.vcf> -genotypeMergeOptions PRIORITIZE -priority Gatk,Sam --filteredrecordsmergetype KEEP_UNCONDITIONAL -o <Snp_Indel_Filt.vcf>

Annotate with SnpEff

java -Xmx4G -jar snpEff.jar eff -c snpEff.config -v CanFam3Broad <Snp_Indel_Filt.vcf> > $<Snp_Indel_Filt_snpEff.vcf>

9.2.3 Pathogen Prediction

Filter PGx variants and select Mis-sense, Frame-shift, Stop-gain/lost, Splice region

java -Xmx2g -jar <GenomeAnalysisTK.jar> -R <canFam3.1> -T SelectVariants <Snp_Indel_Filt_snpEff.vcf> --variant -o <Snp_Indel_Filt_snpEff_PGx.vcf> -L PGx_ORF_Genes.bed

cat <Snp_Indel_Filt_snpEff_PGx.vcf> | snpEff/scripts/vcfEffOnePerLine.pl |java -jar <SnpSift.jar> filter "(EFF[*].EFFECT = 'frameshift_variant')" - > <PGx_ORF_FS.vcf>

cat <Snp_Indel_Filt_snpEff_PGx.vcf> | snpEff/scripts/vcfEffOnePerLine.pl |java -jar <SnpSift.jar> filter "(EFF[*].EFFECT != 'frameshift_variant') & (EFF[*].IMPACT = 'HIGH')" - > <PGx_ORF_HIGH.vcf>

cat <Snp_Indel_Filt_snpEff_PGx.vcf> |snpEff/scripts/vcfEffOnePerLine.pl |java -jar <SnpSift.jar> filter "(EFF[*].EFFECT ='missense_variant')" - > <PGx_ORF_NS.vcf>

9.3 **Tables**

Table 1. The PGx annotation table for all Canine human orthologues

Human Gene	Importance	Type	Canine Gene	Mapping
ABCA1	Transporter	Extended	ABCA1	One2one
ABCA4	Transporter	Extended	ABCA4	One2one
ABCB1	Transporter	Core	MDR1	One2one
ABCB11	Transporter	Extended	ABCB11	One2one
ABCB4	Transporter	Extended	ABCB4	One2one
ABCB5	Transporter	Extended	ABCB5	One2one
ABCB6	Transporter	Extended	ABCB6	One2one
ABCB7	Transporter	Extended	ABCB7	One2one
ABCB8	Transporter	Extended	ABCB8	One2one
ABCC1	Transporter	Extended	MRP1	One2one
ABCC10	Transporter	Extended	ABCC10	One2one
ABCC11	Transporter	Extended	ABCC11	One2one
ABCC12	Transporter	Extended	ABCC12	One2one
ABCC2	Transporter	Core	MRP2	One2one
ABCC3	Transporter	Extended	ABCC3	One2one
ABCC4	Transporter	Extended	ABCC4	One2one
ABCC5	Transporter	Extended	ABCC5	One2one
ABCC6	Transporter	Extended	ABCC6	One2one
ABCC8	Transporter	Extended	ABCC8	One2one
ABCC9	Transporter	Extended	ABCC9	One2one
ABCD1	Unknown	Related	ABCD1	One2one
ABCG1	Transporter	Extended	ABCG1	One2one
ABCG2	Transporter	Core	ABCG2	One2one
ABL1	Unknown	Reltated	ABL1	One2one
ACAA1	Unknown	Reltated	ACAA1	One2one
ACAA2	Unknown	Reltated	ACAA2	One2one
ACAD10	Unknown	Reltated	ACAD10	One2one
ACAD11	Unknown	Reltated	ACAD11	One2one
ACAD8	Unknown	Reltated	ACAD8	One2one
ACAD9	Unknown	Reltated	ACAD9	One2one
ACADL	Unknown	Reltated	ACADL	One2one

ACADM	Unknown	Reltated	ACADM	One2one
ACADS	Unknown	Reltated	ACADS	One2one
ACADSB	Unknown	Reltated	ACADSB	One2one
ACADVL	Unknown	Reltated	ACADVL	One2one
ACAT1	Unknown	Reltated	ACAT1	One2one
ACAT2	Unknown	Reltated	ACAT2	One2one
ACE	Unknown	Core	ENSCAFG00000012998	One2many
ACOT8	Unknown	Reltated	ACOT8	One2one
ACOX1	Unknown	Reltated	ACOX1	One2one
ACOX2	Unknown	Reltated	ACOX2	One2one
ACOX3	Unknown	Reltated	ACOX3	One2one
ADD1	Unknown	Reltated	ADD1	One2one
ADH1A	Phase-I	Core	ENSCAFG00000010410	One2many
ADH1B	Phase-I	Core	ENSCAFG00000010410	One2many
ADH1C	Phase-I	Core	ENSCAFG00000010410	One2many
ADH4	Phase-I	Extended	ADH4	One2one
ADH5	Phase-I	Extended	ADH5	One2many
ADH5	Phase-I	Extended	ENSCAFG00000009465	One2many
ADH5	Phase-I	Extended	ENSCAFG00000013007	One2many
ADH6	Phase-I	Extended	ADH6	One2one
ADH7	Phase-I	Extended	ENSCAFG00000029473	Many2many
ADHFE1	Phase-I	Extended	ADHFE1	One2one
ADORA2A	Unknown	Reltated	ADORA2A	One2one
ADRB1	Unknown	Core	ADRB1	One2one
ADRB2	Unknown	Core	ADRB2	One2one
AHR	Modifier	Core	AHR	One2one
AKAP9	Target/Receptor	Related	AKAP9	One2one
AKR1A1	Unknown	Reltated	AKR1A1	One2one
AKR1B1	Unknown	Reltated	AKR1B1	One2many
AKR1B1	Unknown	Reltated	ENSCAFG00000003343	One2many
AKR1C3	Unknown	Reltated	AKR1C3	One2many
AKR1D1	Unknown	Reltated	ENSCAFG00000028750	One2many
AKR1D1	Unknown	Reltated	ENSCAFG00000029647	One2many
AKR1D1	Unknown	Reltated	ENSCAFG00000032319	One2many
AKR1E2	Unknown	Reltated	AKR1E2	One2one

AKR7L	Unknown	Reltated	AKR7L	One2one
AKT1	Unknown	Reltated	AKT1	One2one
ALB	Target/Receptor	Related	ALB	One2one
ALDH1A1	Phase-I	Core	ALDH1A1	One2one
ALDH1A2	Phase-I	Extended	ALDH1A2	One2one
ALDH1A3	Phase-I	Extended	ALDH1A3	One2one
ALDH1B1	Phase-I	Extended	ALDH1B1	One2one
ALDH2	Phase-I	Extended	ENSCAFG00000008683	Many2many
ALDH2	Phase-I	Extended	ENSCAFG00000019771	Many2many
ALDH3A1	Phase-I	Extended	ALDH3A1	One2one
ALDH3A2	Phase-I	Extended	ALDH3A2	One2one
ALDH3B1	Phase-I	Extended	ALDH3B1	One2one
ALDH3B2	Phase-I	Extended	ALDH3B2	One2one
ALDH4A1	Phase-I	Extended	TAS1R2	One2one
ALDH5A1	Phase-I	Extended	ALDH5A1	One2one
ALDH6A1	Phase-I	Extended	ALDH6A1	One2one
ALDH7A1	Phase-I	Extended	ALDH7A1	One2one
ALDH8A1	Phase-I	Extended	ALDH8A1	One2one
ALDH9A1	Phase-I	Extended	ALDH9A1	One2one
ALK	Unknown	Reltated	ALK	One2one
ALOX5	Unknown	Core	ALOX5	One2one
AMACR	Unknown	Reltated	AMACR	One2one
ANKK1	Unknown	Reltated	ANKK1	One2one
AOX1	Phase-I	Extended	AOH2	One2many
AOX1	Phase-I	Extended	ENSCAFG00000030427	One2many
APOA2	Target/Receptor	Related	APOA2	One2one
APOE	Unknown	Reltated	APOE	One2one
ARG1	Unknown	Reltated	ARG1	One2one
ARNT	Modifier	Extended	ARNT	One2one
ARSA	Modifier	Extended	ARSA	One2one
ARVCF	Target/Receptor	Related	ARVCF	One2one
ASL	Unknown	Reltated	ENSCAFG00000010332	One2many
ASNA1	Target/Receptor	Related	ENSCAFG00000002783	One2many
ASNA1	Target/Receptor	Related	ENSCAFG00000017208	One2many
ASS1	Unknown	Reltated	ASS1	One2one

ATIC	Unknown	Reltated	ATIC	One2one
ATP7A	Modifier	Extended	ATP7A	One2one
ATP7B	Modifier	Extended	ATP7B	One2one
AhRR	Unknown	Reltated	AHRR	One2one
BCR	Unknown	Reltated	BCR	One2one
BDH2	Target/Receptor	Related	BDH2	One2one
BLVRB	Unknown	Reltated	BLVRB	One2one
BRAF	Unknown	Reltated	BRAF	One2one
BRCA1	Unknown	Core	BRCA1	One2one
C11orf65	Unknown	Reltated	C11orf65	One2one
CALU	Unknown	Reltated	CALU	One2one
CAT	Modifier	Extended	CAT	One2many
CAT	Modifier	Extended	ENSCAFG00000002508	One2many
CBR1	Phase-I	Extended	ENSCAFG00000014444	One2many
CBR1	Phase-I	Extended	ENSCAFG00000023660	One2many
CBR1	Phase-I	Extended	ENSCAFG00000031486	One2many
CBR3	Phase-I	Extended	CBR3	One2one
CCR5	Unknown	Reltated	CCR5	One2one
CDA	Modifier	Extended	CDA	One2one
CEBPA	Unknown	Reltated	CEBPA	One2one
CEBPB	Unknown	Reltated	CEBPB	One2one
CES1	Phase-I	Extended	CESDD1	One2one
CES2	Phase-I	Extended	CES2	One2one
CFTR	Modifier	Core	CFTR	One2one
CHRNA2	Target/Receptor	Related	CHRNA2	One2one
CHST1	Phase-II	Extended	CHST1	One2one
CHST10	Phase-II	Extended	CHST10	One2one
CHST11	Phase-II	Extended	CHST11	One2one
CHST12	Phase-II	Extended	CHST12	One2one
CHST2	Phase-II	Extended	CHST2	One2one
CHST3	Phase-II	Extended	CHST3	One2one
CHST4	Phase-II	Extended	CHST4	One2one
CHST5	Phase-II	Extended	ENSCAFG00000020075	One2many
CHST6	Phase-II	Extended	ENSCAFG00000020075	One2many
CHST7	Phase-II	Extended	CHST7	One2one

CHST8	Phase-II	Extended	CHST8	One2one
CHST9	Phase-II	Extended	CHST9	One2one
COL18A1	Target/Receptor	Related	COL18A1	One2one
COL22A1	Unknown	Reltated	COL22A1	One2one
COMT	Target/Receptor	Core	COMT	One2one
COQ2	Unknown	Reltated	COQ2	One2one
CPS1	Unknown	Reltated	CPS1	One2one
CRAT	Unknown	Reltated	CRAT	One2one
CRHR1	Unknown	Reltated	CRHR1	One2one
CRHR2	Unknown	Reltated	CRHR2	One2one
CROT	Target/Receptor	Related	CROT	One2one
CRP	Unknown	Reltated	CRP	One2one
CRYZ	Target/Receptor	Related	CRYZ	One2many
CRYZ	Target/Receptor	Related	ENSCAFG00000002181	One2many
CTSK	Target/Receptor	Related	CTSK	One2one
CYB5R1	Unknown	Reltated	CYB5R1	One2one
CYB5R2	Unknown	Reltated	CYB5R2	One2one
CYB5R3	Phase-I	Extended	CYB5R3	One2one
CYB5R4	Unknown	Reltated	CYB5R4	One2one
CYP11A1	Phase-I	Extended	CYP11A1	One2one
CYP11B1	Phase-I	Extended	ENSCAFG00000001285	One2many
CYP11B2	Phase-I	Extended	ENSCAFG00000001285	One2many
CYP17A1	Phase-I	Extended	CYP17A1	One2one
CYP19A1	Phase-I	Extended	CYP19A1	One2one
CYP1A1	Phase-I	Core	CYP1A1	One2one
CYP1A2	Phase-I	Core	CYP1A2	One2one
CYP1B1	Phase-I	Extended	CYP1B1	One2one
CYP20A1	Phase-I	Extended	CYP20A1	One2one
CYP21A2	Phase-I	Extended	CYP21	One2one
CYP24A1	Phase-I	Extended	CYP24A1	One2one
CYP26A1	Phase-I	Extended	CYP26C1	One2one
CYP27A1	Phase-I	Extended	CYP27A1	One2one
CYP27B1	Phase-I	Extended	CYP27B1	One2one
CYP2A13	Phase-I	Extended	CYP2A13	Many2many
CYP2A6	Phase-I	Core	CYP2A13	Many2many

CYP2A7	Phase-I	Extended	CYP2A13	Many2many
CYP2B6	Phase-I	Core	CYP2B6	One2one
CYP2C18	Phase-I	Extended	ENSCAFG00000013311	Many2many
CYP2C8	Phase-I	Core	CYP2C21	One2many
CYP2E1	Phase-I	Core	CYP2E1	One2one
CYP2F1	Phase-I	Extended	CYP2F1	One2one
CYP2J2	Phase-I	Core	CYP2J2	One2one
CYP2R1	Phase-I	Extended	CYP2R1	One2one
CYP2S1	Phase-I	Extended	CYP2S1	One2one
CYP2W1	Unknown	Reltated	CYP2W1	One2one
CYP39A1	Phase-I	Extended	CYP39A1	One2one
CYP3A4	Phase-I	Core	CYP3A12	Many2many
CYP3A4	Phase-I	Core	CYP3A26	Many2many
CYP3A4	Phase-I	Core	ENSCAFG00000014939	Many2many
CYP3A4	Phase-I	Core	ENSCAFG00000014990	Many2many
CYP3A43	Phase-I	Extended	CYP3A12	Many2many
CYP3A43	Phase-I	Extended	CYP3A26	Many2many
CYP3A43	Phase-I	Extended	ENSCAFG00000014939	Many2many
CYP3A43	Phase-I	Extended	ENSCAFG00000014990	Many2many
CYP3A5	Phase-I	Core	CYP3A12	Many2many
CYP3A5	Phase-I	Core	CYP3A26	Many2many
CYP3A5	Phase-I	Core	ENSCAFG00000014939	Many2many
CYP3A5	Phase-I	Core	ENSCAFG00000014990	Many2many
CYP3A7	Phase-I	Extended	CYP3A12	Many2many
CYP3A7	Phase-I	Extended	CYP3A26	Many2many
CYP3A7	Phase-I	Extended	ENSCAFG00000014939	Many2many
CYP3A7	Phase-I	Extended	ENSCAFG00000014990	Many2many
CYP46A1	Phase-I	Extended	CYP46A1	One2one
CYP4A11	Phase-I	Extended	CYP4A37	Many2many
CYP4A11	Phase-I	Extended	CYP4A38	Many2many
CYP4A11	Phase-I	Extended	CYP4A39	Many2many
CYP4A11	Phase-I	Extended	ENSCAFG00000023399	Many2many
CYP4A22	Unknown	Reltated	CYP4A37	Many2many
CYP4A22	Unknown	Reltated	CYP4A38	Many2many
CYP4A22	Unknown	Reltated	CYP4A39	Many2many

CYP4A22	Unknown	Reltated	ENSCAFG00000023399	Many2many
CYP4B1	Phase-I	Extended	CYP4B1	One2one
CYP4F12	Phase-I	Extended	ENSCAFG00000015931	One2many
CYP4F3	Phase-I	Extended	CYP4F3	One2one
CYP51A1	Phase-I	Extended	CYP51A1	One2one
CYP7A1	Phase-I	Extended	CYP7A1	One2one
CYP7B1	Phase-I	Extended	CYP7B1	One2one
CYP8B1	Phase-I	Extended	CYP8B1	One2one
DDO	Phase-I	Extended	DDO	One2one
DECR1	Unknown	Reltated	DECR1	One2one
DHRS1	Phase-I	Extended	DHRS1	One2one
DHRS13	Phase-I	Extended	ENSCAFG00000008572	Many2many
DHRS2	Phase-I	Extended	DHRS2	One2one
DHRS3	Phase-I	Extended	DHRS3	One2one
DHRS4	Phase-I	Extended	DHRS4	One2many
DHRS4L2	Phase-I	Extended	DHRS4	One2many
DHRS7	Phase-I	Extended	DHRS7	One2one
DHRS7B	Phase-I	Extended	DHRS7B	One2one
DHRS7C	Phase-I	Extended	DHRS7C	One2one
DHRS9	Phase-I	Extended	DHRS9	One2one
DHRSX	Phase-I	Extended	DHRSX	One2one
DNTTIP2	Target/Receptor	Related	DNTTIP2	One2one
DPEP1	Phase-I	Extended	DPEP1	One2one
DPYD	Phase-I	Core	DPYD	One2one
DRD2	Unknown	Core	DRD2	One2one
EAF2	Target/Receptor	Related	EAF2	One2one
ECH1	Unknown	Reltated	ECH1	One2one
ECHS1	Unknown	Reltated	ECHS1	One2one
ECI1	Unknown	Reltated	ECI1	One2one
ECI2	Unknown	Reltated	ECI2	One2one
EGF	Unknown	Reltated	EGF	One2one
EGFR	Unknown	Core	EGFR	One2one
EHHADH	Unknown	Reltated	EHHADH	One2one
EPHX1	Phase-I	Extended	EPHX1	One2one
EPHX2	Phase-I	Extended	EPHX2	One2one

ERBB2	Unknown	Reltated	ERBB2	One2one
ERCC1	Unknown	Reltated	ERCC1	One2one
ESR1	Unknown	Reltated	ESR1	One2one
ESR2	Unknown	Reltated	ESR2	One2one
EXOC6	Target/Receptor	Related	EXOC6	One2one
F2	Unknown	Reltated	F2	One2one
F5	Unknown	Core	F5	One2one
FCER1G	Target/Receptor	Related	FCER1G	One2one
FCGR3A	Unknown	Reltated	ENSCAFG00000013015	One2many
FDPS	Unknown	Reltated	FDPS	One2one
FGFR1	Unknown	Reltated	FGFR1	One2one
FGFR3	Unknown	Reltated	FGFR3	One2one
FIP1L1	Unknown	Reltated	FIP1L1	One2one
FKBP1A	Unknown	Reltated	ENSCAFG00000006795	One2many
FLOT1	Unknown	Reltated	FLOT1	One2one
FLT1	Unknown	Reltated	FLT1	One2one
FLT4	Unknown	Reltated	FLT4	One2one
FMO1	Phase-I	Extended	FMO1	One2one
FMO2	Phase-I	Extended	FMO2	One2one
FMO3	Phase-I	Extended	FMO3	One2one
FMO4	Phase-I	Extended	FMO4	One2one
FMO5	Phase-I	Extended	FMO5	One2one
FMO6P	Phase-I	Extended	FMO6P	One2one
FOXA3	Unknown	Reltated	FOXA3	One2one
G6PD	Target/Receptor	Core	G6PD	One2one
GBA	Unknown	Reltated	GBA	One2one
GCDH	Unknown	Reltated	GCDH	One2one
GCLC	Target/Receptor	Related	GCLC	One2one
GCLM	Target/Receptor	Related	GCLM	One2one
GGCX	Unknown	Reltated	GGCX	One2one
GNB3	Unknown	Reltated	GNB3	One2one
GPLD1	Target/Receptor	Related	GPLD1	One2one
GPX1	Phase-I	Extended	GPX1	One2one
GPX2	Phase-I	Extended	GPX2	One2one
GPX3	Phase-I	Extended	GPX3	One2one

GPX5	Phase-I	Extended	GPX5	One2one
GPX6	Phase-I	Extended	GPX6	One2one
GPX7	Phase-I	Extended	GPX7	One2one
GRIK4	Unknown	Reltated	GRIK4	One2one
GSR	Phase-I	Extended	GSR	One2one
GSS	Phase-I	Extended	GSS	One2one
GSTA1	Phase-II	Extended	ENSCAFG00000002230	One2many
GSTA2	Phase-II	Extended	ENSCAFG00000024271	Many2many
GSTA3	Phase-II	Extended	ENSCAFG00000031682	Many2many
GSTA4	Phase-II	Extended	ENSCAFG00000009164	One2many
GSTA4	Phase-II	Extended	GSTA4	One2many
GSTA5	Phase-II	Extended	ENSCAFG00000002230	One2many
GSTCD	Phase-II	Extended	GSTCD	One2one
GSTK1	Phase-II	Extended	ENSCAFG00000004632	One2many
GSTK1	Phase-II	Extended	ENSCAFG00000023764	One2many
GSTK1	Phase-II	Extended	ENSCAFG00000029004	One2many
GSTM1	Phase-II	Core	ENSCAFG00000019812	One2many
GSTM2	Phase-II	Extended	ENSCAFG00000019812	One2many
GSTM3	Phase-II	Extended	GSTM3	One2one
GSTM4	Phase-II	Extended	ENSCAFG00000019812	One2many
GSTM5	Phase-II	Extended	ENSCAFG00000019812	One2many
GSTO1	Phase-II	Extended	GSTO1	One2one
GSTO2	Phase-II	Extended	GSTO2	One2one
GSTP1	Phase-II	Core	ENSCAFG00000010648	One2many
GSTP1	Phase-II	Core	ENSCAFG00000011452	One2many
GSTP1	Phase-II	Core	ENSCAFG00000019307	One2many
GSTP1	Phase-II	Core	ENSCAFG00000025332	One2many
GSTP1	Phase-II	Core	ENSCAFG00000032752	One2many
GSTP1	Phase-II	Core	GSTP1	One2many
GSTT1	Phase-II	Core	GSTT1	One2one
GSTT2	Phase-II	Extended	ENSCAFG00000014043	One2many
GSTZ1	Phase-II	Extended	GSTZ1	One2one
HADH	Unknown	Reltated	HADH	One2one
HADHA	Unknown	Reltated	HADHA	One2one
HADHB	Unknown	Reltated	ENSCAFG00000004314	One2many

HADHB	Unknown	Reltated	ENSCAFG00000029395	One2many
HAGH	Phase-I	Extended	HAGH	One2one
HIF1A	Unknown	Reltated	HIF1A	One2one
HLA-DOB	Target/Receptor	Related	DLA-DOB	One2one
HLA-DQA1	Unknown	Reltated	DLA-DQA	One2many
HLA-DRB1	Unknown	Reltated	DLA-DRB1	One2many
HMGCR	Target/Receptor	Core	HMGCR	One2one
HNF1A	Unknown	Reltated	HNF1	One2one
HNF4A	Modifier	Extended	HNF4A	One2one
HNMT	Phase-II	Extended	HNMT	One2one
HPRT1	Unknown	Reltated	HPRT	One2one
HSD11B1	Phase-I	Extended	HSD11B1	One2one
HSD17B10	Unknown	Reltated	HSD17B10	One2one
HSD17B14	Phase-I	Extended	HSD17B14	One2one
HSD17B4	Unknown	Reltated	HSD17B4	One2one
HTR1A	Unknown	Reltated	HTR1A	One2one
HTR2C	Unknown	Reltated	HTR2C	One2one
IAPP	Modifier	Extended	IAPP	One2one
IFNL3	Unknown	Reltated	ENSCAFG00000005588	One2many
IGF2R	Target/Receptor	Related	CI-MPR/IGF2R	One2one
IL1B	Unknown	Reltated	IL1B	One2one
IL1RN	Unknown	Reltated	IL1RN	One2one
IL2RA	Unknown	Reltated	IL2RA	One2one
IL6	Unknown	Reltated	IL6	One2one
IL6R	Unknown	Reltated	IL6R	One2one
IL6ST	Unknown	Reltated	IL6ST	One2one
INMT	Unknown	Reltated	ENSCAFG00000013528	Many2many
INTS12	Target/Receptor	Related	INTS12	One2one
ITK	Unknown	Reltated	ITK	One2one
ITPA	Unknown	Reltated	ITPA	One2one
KCNH2	Unknown	Core	KCNH2	One2one
KCNJ11	Modifier	Core	KCNJ11	One2one
KDR	Unknown	Reltated	KDR	One2one
KIT	Unknown	Reltated	KIT	One2one
KRAS	Unknown	Reltated	K-RAS	One2one

LCK	Unknown	Reltated	LCK	One2one
LDLR	Unknown	Reltated	LDLR	One2one
LTC4S	Unknown	Reltated	LTC4S	One2one
MAOA	Target/Receptor	Related	MAOA	One2one
MAOB	Target/Receptor	Related	MAOB	One2one
MAP2K1	Unknown	Reltated	MAP2K1	One2one
MAP2K2	Unknown	Reltated	MAP2K2	One2one
MAT1A	Modifier	Extended	MAT1A	One2one
METAP1	Phase-I	Extended	METAP1	One2one
MGST1	Phase-II	Extended	MGST1	One2one
MGST2	Phase-II	Extended	MGST2	One2one
MGST3	Phase-II	Extended	MGST3	One2many
MKI67	Unknown	Reltated	MKI67	One2one
MPO	Modifier	Extended	MPO	One2one
MS4A1	Unknown	Reltated	MS4A1	One2one
MTHFR	Unknown	Core	MTHFR	One2one
MTOR	Unknown	Reltated	MTOR	One2one
MTRR	Unknown	Reltated	MTRR	One2one
NAGS	Unknown	Reltated	NAGS	One2one
NCOA1	Unknown	Reltated	NCOA1	One2one
NFE2L2	Target/Receptor	Related	NFE2L2	One2one
NFKB1	Unknown	Reltated	NFKB1	One2one
NHLRC1	Target/Receptor	Related	NHLRC1	One2one
NNMT	Phase-II	Extended	NNMT	One2one
NOS1	Phase-I	Extended	NOS1	One2one
NOS3	Phase-I	Extended	NOS3	One2one
NQO1	Target/Receptor	Core	NQO1	One2one
NR0B2	Unknown	Reltated	NR0B2	One2one
NR1H2	Unknown	Reltated	NR1H2	One2one
NR1H3	Unknown	Reltated	NR1H3	One2one
NR1H4	Unknown	Reltated	NR1H4	One2one
NR1I2	Modifier	Core	PXR	One2one
NR1I3	Modifier	Extended	CAR	One2one
NR3C1	Target/Receptor	Related	NR3C1	One2one
NR5A2	Unknown	Reltated	NR5A2	One2one

ORM1	Target/Receptor	Related	ENSCAFG00000003331	One2many
ORM2	Target/Receptor	Related	ENSCAFG00000003331	One2many
OTC	Unknown	Reltated	OTC	One2one
P2RY1	Unknown	Core	P2RY1	One2one
P2RY12	Unknown	Core	P2RY12	One2one
PDE3A	Phase-I	Extended	PDE3A	One2one
PDE3B	Phase-I	Extended	PDE3B	One2one
PDGFRA	Unknown	Reltated	PDGFRA	One2one
PDGFRB	Unknown	Reltated	PDGFRB	One2one
PGR	Unknown	Reltated	PGR	One2one
PIK3CA	Unknown	Reltated	PIK3CA	One2one
PKD2	Target/Receptor	Related	PKD2	One2one
PLG	Target/Receptor	Related	PLG	One2one
PML	Unknown	Reltated	PML	One2one
PNMT	Phase-II	Extended	PNMT	One2one
POLG	Unknown	Reltated	POLG	One2one
PON1	Phase-I	Extended	PON1	One2one
PON2	Phase-I	Extended	PON2	One2one
PON3	Phase-I	Extended	PON3	One2one
POR	Modifier	Extended	OR	One2one
PPARA	Modifier	Extended	PPARA	One2one
PPARD	Modifier	Extended	PPARD	One2one
PPARG	Modifier	Extended	PPARG	One2one
PPP1R9A	Target/Receptor	Related	PPP1R9A	One2one
PRKAB2	Target/Receptor	Related	PRKAB2	One2one
PSMB8	Target/Receptor	Related	PSMB8	One2one
PTGIS	Target/Receptor	Core	PTGIS	One2one
PTGS2	Unknown	Core	COX-2	One2one
RALBP1	Target/Receptor	Related	RALBP1	One2one
RARA	Unknown	Reltated	RARA	One2one
RHD	Unknown	Reltated	RH30	One2many
RPS6KB1	Unknown	Reltated	RPS6KB1	One2one
RXRA	Modifier	Extended	RXRALPHA	One2one
RYR1	Unknown	Core	RYR1	One2one
SAA1	Unknown	Reltated	SAA1	Many2many

SCN1A	Unknown	Reltated	SCN1A	One2one
SCN5A	Unknown	Core	SCN5A	One2one
SCP2	Unknown	Reltated	SCP2	One2one
SERPINA7	Modifier	Extended	TBG	One2one
SERPINC1	Unknown	Reltated	SERPINC1	One2one
SGOL2	Target/Receptor	Related	SGOL2	One2one
SHBG	Target/Receptor	Related	SHBG	One2one
SLC10A1	Transporter	Extended	SLC10A1	One2one
SLC10A2	Transporter	Extended	SLC10A2	One2one
SLC13A1	Transporter	Extended	SLC13A1	One2one
SLC13A2	Transporter	Extended	SLC13A2	One2one
SLC13A3	Transporter	Extended	SLC13A3	One2one
SLC15A1	Transporter	Extended	SLC15A1	One2one
SLC15A2	Transporter	Core	SLC15A2	One2one
SLC16A1	Transporter	Extended	MCT1	One2one
SLC19A1	Transporter	Core	SLC19A1	One2one
SLC22A1	Transporter	Core	SLC22A1	One2one
SLC22A10	Transporter	Extended	SLC22A10	One2one
SLC22A11	Transporter	Extended	SLC22A11	One2one
SLC22A12	Transporter	Extended	SLC22A12	One2one
SLC22A13	Transporter	Extended	SLC22A13	One2one
SLC22A14	Transporter	Extended	SLC22A14	One2one
SLC22A15	Transporter	Extended	SLC22A15	One2one
SLC22A16	Transporter	Extended	SLC22A16	One2one
SLC22A17	Transporter	Extended	SLC22A17	One2one
SLC22A18	Transporter	Extended	SLC22A18	One2one
SLC22A2	Transporter	Core	SLC22A2	One2one
SLC22A3	Transporter	Extended	SLC22A3	One2one
SLC22A4	Transporter	Extended	SLC22A4	One2one
SLC22A5	Transporter	Extended	SLC22A5	One2one
SLC22A6	Transporter	Core	SLC22A6	One2one
SLC22A7	Transporter	Extended	SLC22A7	One2one
SLC22A8	Transporter	Extended	SLC22A8	One2one
SLC22A9	Transporter	Extended	ENSCAFG00000015226	One2many
SLC27A1	Transporter	Extended	SLC27A1	One2one

SLC28A1	Transporter	Extended	SLC28A1	One2one
SLC29A2	Transporter	Extended	SLC29A2	One2one
SLC2A4	Transporter	Extended	SLC2A4	One2one
SLC2A5	Transporter	Extended	SLC2A5	One2one
SLC47A1	Unknown	Reltated	SLC47A1	One2one
SLC47A2	Unknown	Reltated	SLC47A2	One2one
SLC5A6	Transporter	Extended	SLC5A6	One2one
SLC6A6	Transporter	Extended	SLC6A6	One2one
SLC7A5	Transporter	Extended	SLC7A5	One2one
SLC7A7	Transporter	Extended	SLC7A7	One2one
SLC7A8	Transporter	Extended	SLC7A8	One2one
SLCO1A2	Transporter	Extended	OATPA	One2one
SLCO1B1	Transporter	Core	OATPC	One2many
SLCO1B3	Transporter	Core	OATPC	One2many
SLCO1B7	Unknown	Reltated	OATPC	One2many
SLCO1C1	Transporter	Extended	SLCO1C1	One2one
SLCO2A1	Transporter	Extended	SLCO2A1	One2one
SLCO2B1	Transporter	Extended	SLCO2B1	One2one
SLCO3A1	Transporter	Extended	SLCO3A1	One2one
SLCO4A1	Transporter	Extended	SLCO4A1	One2one
SLCO4C1	Transporter	Extended	SLCO4C1	One2one
SLCO5A1	Transporter	Extended	SLCO5A1	One2one
SLCO6A1	Transporter	Extended	SLCO6A1	One2one
SOD1	Modifier	Extended	SOD1	One2one
SOD2	Modifier	Extended	SOD2	One2one
SOD3	Modifier	Extended	SOD3	One2one
SPG7	Target/Receptor	Related	SPG7	One2one
STK19	Target/Receptor	Related	STK19	One2one
SULF1	Phase-I	Extended	SULF1	One2one
SULT1A1	Phase-II	Core	SULT1A1	One2many
SULT1A2	Phase-II	Extended	SULT1A1	One2many
SULT1A3	Phase-II	Extended	SULT1A1	One2many
SULT1B1	Phase-II	Extended	SULT1B1	One2one
SULT1C2	Phase-II	Extended	SULT1C2	One2one
SULT1E1	Phase-II	Extended	SULT1E1	One2one

SULT2A1	Phase-II	Extended	ENSCAFG00000000894	Many2many
SULT2B1	Phase-II	Extended	SULT2B1	One2one
SULT4A1	Phase-II	Extended	SULT4A1	One2one
TAP1	Transporter	Extended	TAP1	One2one
TAP2	Transporter	Extended	TAP2	One2many
TBXAS1	Target/Receptor	Related	TBXAS1	One2one
TMEM43	Unknown	Reltated	TMEM43	One2one
TMEM63A	Target/Receptor	Related	TMEM63A	One2one
TNFRSF8	Unknown	Reltated	TNFRSF8	One2one
TNFSF13B	Unknown	Reltated	TNFSF13B	One2one
TNIP1	Target/Receptor	Related	TNIP1	One2one
TOMM40L	Target/Receptor	Related	TOMM40L	One2one
TP53	Unknown	Reltated	P53	One2one
TPMT	Phase-II	Core	TPMT	One2one
TSC1	Unknown	Reltated	TSC1	One2one
TSC2	Unknown	Reltated	TSC2	One2one
TSPO	Target/Receptor	Related	TSPO	One2one
TTBK1	Target/Receptor	Related	TTBK1	One2one
TTR	Target/Receptor	Related	TTR	One2one
TYMS	Target/Receptor	Core	TS	One2one
UGT1A10	Phase-II	Extended	ENSCAFG00000031066	One2many
UGT1A4	Phase-II	Extended	ENSCAFG00000024836	Many2many
UGT1A6	Phase-II	Extended	UGT1A6	One2one
UGT1A7	Phase-II	Extended	ENSCAFG00000031066	One2many
UGT1A9	Phase-II	Extended	ENSCAFG00000031066	One2many
UGT2A1	Phase-II	Extended	ENSCAFG00000002857	One2many
UGT2B10	Phase-II	Extended	ENSCAFG00000031254	Many2many
UGT2B11	Phase-II	Extended	ENSCAFG00000030088	Many2many
UGT2B15	Phase-II	Core	ENSCAFG00000028888	Many2many
UGT2B17	Phase-II	Core	ENSCAFG00000029376	Many2many
UGT2B28	Phase-II	Extended	UGT2B31	Many2many
UGT2B4	Phase-II	Extended	ENSCAFG00000030088	Many2many
UGT2B7	Phase-II	Core	ENSCAFG00000030088	Many2many
UGT8	Phase-II	Extended	UGT8	One2one
UMPS	Unknown	Reltated	UMPS	One2one

UNC93B1	Target/Receptor	Related	ENSCAFG00000011210	One2many
UROC1	Target/Receptor	Related	UROC1	One2one
USF1	Unknown	Reltated	USF1	One2one
VDR	Unknown	Core	VDR	One2one
VEGFA	Unknown	Reltated	VEGFA	One2one
VKORC1	Target/Receptor	Core	VKORC1	One2many
XDH	Phase-I	Extended	XDH	One2one
XRCC1	Unknown	Reltated	XRCC1	One2one
YEATS4	Unknown	CPIC	YEATS4	One2one
ZBED1	Target/Receptor	Related	ENSCAFG00000004548	Many2many