Breast cancer is an umbrella term for encompassing multiple different subtypes, with each subtype having a different biological and clinical features, progression pattern, treatment response and prognosis. Stratification of breast cancer is an important objective, which allows clinicians to better evaluate patient risk and select effective therapeutic strategies based on the subtype in question (Dai et al., 2015).

A cancer type that has met with a myriad of challenges in research efforts is the Triple-negative breast cancer (TNBC). TNBC is a subtype of breast cancer that does not have any of the receptors (the oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)) that are commonly found in breast cancer. Due to its TN status, TNBC is not sensitive to currently used anti-estrogen hormone therapies or HER2-targeted therapy. As such, TNBC leads to more aggressive phenotypes than other breast subtypes with no standardised targeted treatments available. TNBC had a 5-year survival rate 8% to 16% lower than other subtypes (Howard & Olopade, 2021) . Thus, a robust method that assists in differentiating between TNBC and non-TNBC can prove to be useful in the clinical management of breast cancer patients.

Earlier, breast cancer stratification was conventionally performed using a combination of immunohistochemistry (IHC) detection for cellular markers as well as anatomical features such as tumour node size, number of metastases detected, etc. However, information from gene expression profiling by microarrays became an increasingly important means of breast cancer classification (Dai et al., 2015).

It is a well-known issue that there is little consensus in data mined between different omics studies, including microarray gene expression studies, where lack of reproducibility has been brought up as an issue (Draghici et al., 2006; Shi et al., 2008; Sweeney et al., 2017). There could be multiple reasons for this lack of reproducibility. Firstly, the diversity of available microarray platforms with different manufacturing strategies, probe sequences, versions and measurement methods (for example, one channel versus two-channel microarrays), makes it difficult to compare differentially-expressed genes between studies using different platforms. Secondly, technical variation generated during the data generation and collection process introduces unwanted noise into the data, compromising the accuracy, reproducibility and generalizability of the results obtained from that data. An example of such variation are batch effects (Goh et al., 2017). Since such technical variability is dataset-specific, it leads to different datasets being affected by batch effects to different degrees, leading to lack of consistency in the obtained results. Furthermore, even though batch-effect correction algorithms such as ComBat (Johnson et al., 2007) and surrogate variable analysis (SVA) (Leek & Storey, 2007) exist, if they are incorrectly applied, or if used on datasets with unbalanced distribution of batches across classes, they might inadvertently remove relevant variation or add irrelevant variation into the dataset and thereby compromise reproducibility. Thirdly, the properties of certain commonly-used pre-processing methods may introduce biases into the data. For example, the often used robust multichip average (RMA) method of pre-processing data has been shown to dilute biological signal during the quantile normalization (Kim et al., 2014; Zhao et al., 2020) stage as well as during its summarization stage via median polish (Kim et al., 2014).

A fourth reason for lack of reproducibility among different datasets is that compositional differences in the underlying subpopulations comprising the datasets may cause differences in the results generated by each dataset. For example, imagine that we are investigating differentially-expressed genes (DEGs) between diseased and healthy populations. Suppose that the disease under study has two distinct subtypes. Also imagine that the diseased population in dataset 1 contained 70% samples from subtype 1 and 20% samples from subtype 2, while that in dataset 2 contained 20% from subtype 1 and 80% from subtype 2. In this case, the DEGs from dataset 1 would be dominated by those differentiating subtype 1 samples from the healthy samples, while those from dataset 2 would be dominated by those differentiating subtype 2 samples from dataset 2. If the two subtypes are sufficiently different in terms of gene expression profiles and underlying pathological pathways, then there would be very little overlap between the DEG lists obtained from the two datasets. Compositional differences may also arise from factors such as age, gender or race. Finally, another reason for lack of reproducibility is the inherent instability of the p-value metric used to select the DEG list (Halsey et al., 2015).

There have been several proposals to rescue reproducibility from all these forces adversely affecting it. For instance, to overcome the erasure of biological signal due to global quantile normalization in the presence of high class-effect proportion, Zhao et al. (2020) suggested performing quantile normalization in a class-specific manner, so as to preserve the inter-class differences. Sweeney et al. (2017) proposed pooling information from several independent studies by meta-analysis in order to obtain more reproducible lists of DEGs. Zhang et al. (2009) took into consideration correlation within DEG lists rather than just the degree of overlap between DEG lists as a metric for reproducibility. A rank-based normalization method, gene fuzzy scoring (GFS), was proposed to overcome the biases introduced by other normalization methods (Belorkar & Wong, 2016) which compromise reproducibility.

1. **METHODOLOGY**
   1. **Datasets**
      1. **GSE762752 microarray dataset from GEO**

Our main dataset is GSE76275 which is a compilation of microarray data conducted on the platform GPL570 with  Affymetrix Human Genome U133 Plus 2.0 Array. It is a superseries comprising 2 microarray submission, encompassing 198 TNBC tumours and 67 non-TNBC tumours. The dataset is described in the accompanying publication of Burstein et al.  (2015). In this study, the authors have identified and validated 4 subtypes : Luminal-AR (LAR), Mesenchymal (MES), Basal-Like Immune-Suppressed (BLIS), and Basal-Like Immune-Activated (BLIA). Metadata for this dataset was obtained from the GEOquery Bioconductor R package (Davis & Meltzer, 2007). Progesterone receptor (PR) status, estrogen receptor (ER) status, and HER2 amplification status were also provided in the metadata. Raw Affymetrix CEL files were downloaded from the corresponding accession page on GEO.

1. **GSE43358** **microarray dataset from GEO**

Our analyses on the main dataset were subsequently repeated on our validation dataset (GSE43358) comprising microarray-derived gene expression data from a smaller set of 57 samples utilising the same platform and chip. This dataset consisted of 17 TNBC samples and 40 non-TNBC samples. Similar to the original dataset, the metadata was obtained using GEOquery, and the raw CEL files were downloaded from the GEO accession page. PR status, ER status, and HER2 amplification status were also provided in the metadata for this dataset.

Figure 1 below describes our project workflow. In brief, for each dataset, the expression data from the raw CEL files was processed according to the selected pre-processing method, either class-specific quantile normalization (method 1) or GFS (method 2). More details regarding these pre-processing methods are given in the subsequent sections. Following pre-processing, the differential gene expression analysis was carried out. Limma was used for class-specific quantile-normalized data, while the Mann-Whitney U test was performed for the GFS-normalized data. The differentially expressed gene list was obtained for each pre-processing method, for each dataset. The DEG lists from the two datasets were intersected to obtain the percentage of overlapping genes (POG) as defined in (Zhang et al., 2009).

The RMA method consists of three steps: (1) Background subtraction, (2) Quantile normalization and (3) Summarization (Irizarry et al., 2003). In method 1, since quantile normalization was to be carried out separately for the two classes, the raw CEL files for the TNBC and non-TNBC samples were read in separately using the oligo package (Carvalho & Irizarry, 2010).

However, this approach is not sustainable in the case of small datasets containing multiple factors to account for, in which case it would be difficult to maintain the factors balanced across all subgroups. Thus, the accuracy of results obtained after class-specifc quantile normalization heavily depends on the composition of the groups within which normalization was performed. This resembles the issue brought up by Patil et al. (2015) regarding test set bias. Test set bias refers to the phenomenon whereby the classification outcome for a patient sample depends not on its actual class, but on the composition of the accompanying samples it was normalized with (assuming a multi-sample normalization method such as quantile normalization). Even though what we are attempting in this report is not exactly a classification task, similar principles apply. As we saw in section ­3.1, the composition of the non-TNBC samples in both datasets is not similar. Thus, it is not entirely surprising that the DEG lists between the two datasets are not concordant. However, Patil et al. also bring up an interesting point: “A gene signature that employs rank-based features or makes other rank-based calculations is one robust approach to avoiding test set bias.” (Patil et al., 2015). Since GFS generates features purely based on rank, it stands to reason that it would be robust to the differences in composition among datasets 1 and 2.

Another reason why GFS performed better than limma might be because of the inherent instability of the p-value as a metric. As Halsey et al. (2015) point, results relying purely on p-value are not reproducible because a great deal of statistical power is required in order to obtain a truly stable p-value. To generate a stable p-value, one needs an accurate estimate of the mean and variance of gene expression measurement for a particular class, which allows one to determine how likely an observed difference in gene expression values is due to pure chance alone, assuming the null hypothesis is true. However, due to large biological variation within that class, it is not always possible to accurately estimate either of those quantities without very large sample sizes. This is a limitation of methods such as limma, which operate on expression values. On the other hand, since GFS operates purely on ranks, it is less vulnerable to biological sampling variability, and thus more likely to generate more reproducible results across datasets. This is aided by the fact that most of the ranks of the low-expression genes (whose expression values are highly variable) are converted to 0 by GFS, thus downweighting their adverse impact on reproducibility (Belorkar and Wong, 2016).

It must be noted that POG as a metric of reproducibility comes with its own set of limitations, one of which is its value sensitivity to the length of the lists. This leads to variation in interpretation when comparing the effect of normalisation techniques to different datasets. To illustrate, the difference between GSE76275 using GFS and that using cass-specific quantile normalisation (QN) is 0.152 - 0.041, while GSE43358 has a difference of 0.719 - 0.276. Does this mean that the latter dataset is more impacted by the difference in normalisation techniques compared to GSE76275? In other words, do the POG scores reflect the magnitude of obscuring noise in the original data when comparing across datasets? However such conclusions may be erroneous due to the variation in list sizes which affect the POG scores in a probabilistic manner. Here, the QN method is limited in producing sufficient DEGs for POG comparison, leading to a huge reduction in the list size which decreases the probability of finding intersecting genes markedly. In similar light, GFS is effective in producing longer lists which are translated to a higher probability of finding overlaps. Some studies have taken steps to reduce the contribution by probability changes due to length by accounting for list sizes in their POG score normalizations and also considered regulation direction in their definition of gene overlaps (Zhang et al., 2009). Other studies have also improved the POG calculation by accounting for functionally similar genes between the lists (Gong et al., 2010).

Figure 1 below describes our project workflow. In brief, for each dataset, the expression data from the raw CEL files was processed according to the selected pre-processing method, either class-specific QN (method 1) or GFS (method 2). More details regarding these pre-processing methods are given in the subsequent sections. Following pre-processing, differential gene expression analysis was carried out. The Bioconductor package limma (Ritchie et al., 2015). was used for DEG analysis of class-specific QN data, while the Mann-Whitney U test was used for the GFS-normalized data. The differentially expressed gene list was obtained for each pre-processing method, for each dataset. The DEG lists from the two datasets obtained from limma were intersected to obtain the percentage of overlapping genes (POG) for class-specific QN as defined in (Zhang et al., 2009). Similarly, the DEG lists from the two datasets obtained from the Mann-Whitney U test were intersected to obtain the POG for GFS. GO term ORA analysis and PPI analysis was also performed for the obtained DEG lists.

Since the samples in dataset 1 were submitted on two different dates, and since the authors described the samples as being from two different sets (discovery and validation), there was a possibility of batch effects being present in the data. However, since it was unclear as to which factor pertained to batch (submission date or sample set), we decided to use SVA to detect latent variables pertaining to batch. SVA was performed using the Bioconductor “sva” package (Leek et al., 2012). To prevent the biases introduced by QN (whether global or class-specific) from influencing the SVA outcome, the expression matrix fed into SVA was not subjected to QN. However, background subtraction and summarization were carried out as usual before performing SVA.

Following the ORA, the enriched GO terms were summarized into treemap plots using the package rrvgo (Sayols, 2020).

Different data normalisation techniques can potentially present highly distinct interpretations of the biological data. In this aspect, GFS has proven to successfully transform datasets from the same phenotype into similar gene expression matrices by accounting for obscuring noise in measurements while retaining interesting biological variations. In our report, in general, GFS gave superior results to class-specific QN. In terms of reproducibility of DEGs, GFS gave better POG values and GFS-produced DEG lists showed greater intersection size as compared to those from class-specific QN (section 3.3). However, simple gene intersections may not always be the best measure of reproducibility. For instance, when Huang et al. (2018) were investigating the extent of overlap in genes among 33 breast cancer gene signatures, they discovered that while there was little overlap in the genes themselves, the enriched functions obtained from the genes in the signatures showed considerable overlap. To explore the possibility of functional overlap in our own investigation, we also decided to carry out ORA and PPI network analysis.

The use of overlapping GO terms from ORA to evaluate functional consistency may have been a somewhat crude method, since it relies highly on a cut-off (such as p-value and/or effect size) being implemented to get the list of DEGs, which biases the process. More sophisticated methods such as FSNET or PFSNET, which induce subnetworks from known biological networks by using the gene fuzzy score of individual genes as weights, might give better results to evaluate functional consistency in the form of known biological pathways (Lim & Wong, 2014).

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