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Supplementary information

Principles of reproducible metabolite profiling of enriched lymphocytes in tumors and ascites from human ovarian cancer

In the format provided by the authors and unedited

Supplemental Notes 1-5 and Supplemental Method 1

SUBJECT INFORMATION and CONSENT FORM

(insert logo)	***(insert study name)***

Principal Investigator(s): *(insert Principal Investigator(s) information)*

Sponsors: *(insert sponsor(s) information)*

Why are you being asked to participate in this study?
You are being invited to participate because you will soon have a medical procedure (such as surgery, a biopsy, or removal of fluid) to treat an ovarian or other gynecological tumor. You are being asked to donate your tissue (surgical tissue, biopsy tissues, fluids) and clinical information to participate in a research study called *(insert biobank and study name)* to support cancer research.

Your participation in these projects is voluntary and you may decide to participate or to withdraw at any time. Your decision to participate will not affect your treatment in any way and your doctors will continue to provide the best available treatment for your disease. The purpose of this form is to provide you with information about *(insert biobank and study name)* to ask if you will agree to participate.

What is the *(insert biobank name)***?**
It is a biobank that is a collection of tissues and clinical information donated by people who have had a medical procedure such as surgery to treat a tumor. The Biobank provides a resource for a range of cancer research projects at BC Cancer, and also across Canada and internationally. Each donation can support several research projects. The (insert study name) is a specific example of research projects that are supported by the Biobank.

What tissues will the Biobank collect and how will it obtain my tissues?
During the assessment of each specimen removed at the time of a medical procedure, small samples are normally taken by Pathologists in order to make a diagnosis. These tissue samples are then stored in the Department of Pathology for your future clinical care. Excess tissue not needed for your clinical care often remains, both after the procedure when small samples have been taken to make a diagnosis, and then after the diagnosis, from amongst the samples that are taken and stored by Pathologists. The Biobank collects these excess tissues and processes and stores these for research, after the medical procedure and after the Pathologists have determined that they are not required by the hospital for your diagnosis.

What information will the Biobank collect and store?
The Biobank collects information on the tissue, clinical information from the medical record, and personal information that is relevant to the disease. The tissue information includes the composition of the tissue, the size and type of tumor (or, in the case of fluids, the appearance and amount of fluid). The clinical information includes the subject's age, the results of clinical tests such as x-rays, the type of treatment, and follow-up information about the outcome of the treatment. The personal information includes the subject's name, age and symptoms. This information is stored as coded tissue samples and coded paper and computer files in a secure location within the *(insert location)*. The tissue and information will be stored indefinitely and the measures taken by the Biobank to ensure the security and integrity of your donation will be reviewed annually by the *(insert name)* Research Ethics Board.

What will be released to researchers and for what research projects?
Researchers can obtain the materials only if they apply to the Biobank through a formal scientific review process and if they also obtain approval for their research project from the *(insert name)* Research Ethics Board or a properly constituted Research Ethics Board. Research Ethics Boards review research projects to make sure that they follow standards of fairness in protecting the rights of research subjects. The materials provided include tissue, products of the tissue, and information that are coded so that they are anonymous, meaning that they cannot be linked to the individual donor by the researcher or anyone else. The research studies will involve testing tissue samples for features of cells, including the structure and expression of genes, and relating these features to the associated information about the subject and the disease, to gain a better understanding of how tumors develop, grow, spread, and how tumors can be better treated. The studies may also involve testing tissue and fluid samples and analyzing information for teaching, education, and internal program evaluation.

If any other type of research study is proposed, such as one that involves hereditary genetic testing with your tissue (to see if cancer or other diseases run in your family) you will be contacted and asked to give your permission for this type of hereditary genetic testing. These genetic tests will not be done without your permission.

Any study related data and samples, sent outside of Canadian borders may increase the risk of disclosure of information because the laws in those countries dealing with protection of information may not be as strict as in Canada. However, all study data and samples, that might transferred outside of Canada will be coded (this means it will not contain your name or personal identifying information). Any information will be transferred in compliance with all relevant Canadian privacy laws. By signing this consent form, you are consenting to the transfer of your information and samples, to organizations located outside of Canada.

What is the *(insert study name)*?
This is a specific research study to evaluate how the immune system responds to ovarian and other gynecological cancers. It will be conducted using a small part of the tissues and clinical information collected by the Biobank. Blood and tissue samples collected over the course of your treatment will be used to measure changes in the strength of your body's immune response against ovarian and other gynecological cancers over time. Our goal is to better understand the immune response to ovarian

and other gynecological cancers, and devise ways to enhance it in all women, so that we can improve outcomes for this disease.

What will I be asked to do if I agree to participate in the *(Biobank and insert study name)*?

1. No special test or procedure is required to donate your tissue. If there is any excess tissue not needed for your clinical care, then this tissue will be transferred to the Biobank. Excess tissue samples may be related to a current medical procedure or may have been taken at the time of a medical procedure in the past and then stored in the Department of Pathology. Should your tumor recur and require future medical procedures, then excess tissue at this time will also be transferred to the Biobank.

2. Blood will be taken from you several times throughout the course of your treatment and follow-up care. Approximately 200 mL (equivalent to about 14 tablespoons) will be taken from you prior to your medical procedure, upon completion of your treatment, upon recurrence (if applicable), and yearly for up to 10 years. The blood samples will be drawn from a vein in your arm. Every effort will be made to have your blood drawn at the same time as any regularly scheduled tests or appointments with your oncologist or surgeon to avoid an extra needle prick. A study coordinator will contact you to remind you when a blood draw is required about one to two weeks ahead of time. You may choose not to provide a blood sample, and if so you may still continue to participate in the *(Biobank and insert study name).*

3. Information will be collected regarding the diagnosis and type and stage of your tumor, as well as your personal medical history such as your age and symptoms. This information will be collected directly from you and from your medical records. These medical records may include those in your family doctor's office, the hospital, BC Cancer, and laboratories that have participated in your care. As it is important to know the outcome of your treatment, we will also request updates of this information from the same sources in the future.

4. You will be requested to complete a questionnaire. This is optional and you may choose not to complete the questionnaire but to still participate in the Biobank.

What are the risks?

There are no physical risks to you that arise from the collection of a sample of tissue/fluids from your medical procedure. There may be mild pain or discomfort from the collection of your blood sample. The amount of blood taken at any one time for this project should not affect your overall well-being. There may be a risk that your privacy could be compromised. The staff of the Biobank will strive to minimize this risk of loss of privacy by ensuring that your donated samples and data are stored within the Biobank using code numbers and practices that maintain confidentiality and security standards. Samples and data will only be released for research under anonymous code numbers.

What are the benefits?

There is no direct benefit to you from participating in this project. Results obtained from research studies that include samples of your tissue and data will not be given to you or entered into your medical record. The research that will be done with your samples

may help other people in the future who have either the same type of tumor that you have or another type.

What will be done to protect your confidentiality?
Information that links your identity to your samples and your records must be maintained to enable the project to update your record with additional clinical information concerning your status and the outcome of your treatment, and also to enable the project to withdraw your tissue and information if you wish to withdraw your consent in the future. However, this information that links your identity to the sample or the record will not be released to researchers. Identifying information will only be available to the director of the Biobank and members of the Biobank staff, and then only when necessary to update your record. Research records and medical records identifying you may also be inspected by *(insert names)* for the purpose of monitoring research. However, no records that identify you will be allowed to leave the Centre. These organizations have policies of strict confidentiality and the individuals inspecting your records must sign a *(insert name)* confidentiality form (the form is not applicable to Health Canada, or U.S. Food and Drug Administration officials, who have the legal right to inspect health records and are bound to confidentiality by specific laws). Your confidentiality will be respected. No information that discloses your identity will be released or published without your specific consent. Your identity will not be used in any research reports. Records or samples provided to researchers will be identified only by an anonymous code. All information associated with the Biobank will be kept behind locked doors or in secure computer files. The Biobank operates with security standards that include organizational policies and a commitment to privacy that is embodied in all standard operating procedures, physically secure areas within the (*insert location*), and technological measures such as passwords, encryption, and anonymization of data. Your rights to privacy are legally protected by federal and provincial laws that require safeguards to ensure that your privacy is respected and also give you the right of access to the information about you that has been provided to the sponsor and, if need be, an opportunity to correct any errors in this information. Further details about these laws are available on request to the director of the Biobank or the Research Ethics Board.

Will I receive compensation?
You will receive no payment or compensation for your participation in the Biobank. In the long-term, if a diagnostic or therapeutic product or service is developed, you will not receive a financial benefit.

Will anyone else receive remuneration?
No tissue, blood or clinical information is used for commercial purposes. Researchers may be charged a user fee to cover some of the costs of storage and release and operation of the Biobank. Researchers who receive material must agree that no tissues will be sold or used for commercial purposes and will only be used to support cancer research.

The sponsors of this study may reimburse (*insert name*) for all or part of the costs of conducting this. However, neither the *(insert name)* nor any of the investigators or staff conducting this study will receive any personal payments for conducting this study.

How can I withdraw in the future?

You may withdraw from this study at any time without giving reasons. The Biobank will withdraw and destroy all of your tissue and blood samples that remain in the Biobank. We will also destroy all your personal and medical information in our files. We will retain only a record of who had access to your tissue and information, a reference to any studies that have used your tissue and information, and the research data obtained.

Contact:
If you have any questions or desire further information with respect to this study, you may contact:

The Biobank Project Coordinator
Telephone (xxx) xxx-xxxx

The Principle Investigator of the Biobank: *(insert name and contact information)*

The Principal Investigator of the Study: *(insert name and contact information)*.

Or, you can speak to
The *(insert name)* at *(insert organization*):
Telephone (xxx) xxx-xxxx extension xxxx

If you have any concerns about your treatment or rights as a research subject you may contact the Research Participant Complaint Line at the *(insert name and contact information).*

Subject Consent:
My signature on this consent form means:

- I have read and understood the information in this consent form.
- I have had enough time to think about the information provided.
- I have been able to ask for advice if needed.
- I have been able to ask questions and have had satisfactory responses to my questions.
- I understand that all of the information collected will be kept confidential and that the results will only be used for scientific purposes.
- I understand that my participation in this study is voluntary.
- I understand that I am completely free at any time to refuse to participate or to withdraw from this study at any time, and that this will not change the quality of care that I receive.
- If I withdraw, my samples and information in the Biobank will be destroyed.
- I authorize access to my health records and samples as described in this consent form.
- I understand that I am not waiving my legal rights as a result of signing this consent form.
- I understand that there is no guarantee that this study will provide any benefits to me.
- Although I cannot have access to specific test results directly related to my individual tissue samples, I may ask questions about the *(insert study name*) and research being done.
- I will receive a signed consent form copy including all attachments, for my own records.

a. I agree to participate in the Biobank and the use of tissue samples and data collected from me for other research and teaching purposes related to cancer research that will involve testing my tissue sample for features of cells that may provide a better understanding of how tumors develop, grow, spread, and how tumors can be better treated.

   Yes _____(initials) No _____(initials)

b. I agree to participate in the Biobank and the use of blood samples collected from me for research and teaching purposes related to cancer research that will involve testing my tissue sample for features of cells that may provide a better understanding of how tumors develop, grow, spread, and how tumors can be better treated.

   Yes _____ (Initials) No _____ (Initials)

c. I agree to participate in the specific *(insert study name*) that will involve testing my tissue and blood samples for features of the immune response to ovarian or other gynecological cancers.

   Yes _____(initials) No _____(initials)

I will receive a signed copy of this consent form for my own records. I consent to participate in this study.

_____	_____	_____
Subject's Signature	Printed name	Date

_____	_____	_____	_____
Signature of Person Obtaining Consent	Printed name	Study Role	Date

Biobank Tissue Handover: Pathologist to Biobank

Add Biobank Identifier

Tissue Collection

Collected by (initials)		**Collection Date**		**Collection Facility**		**Anesthesia Time**		**Surgery Start Time**		**Incision Time**	
Received by (initials)		**Date Received in Lab**		**Time Received**		**Pathologist**		☐ **Data entered into database Date/Initials**			
Collection Notes											

Label	Tissue Removal Time	Time Biobank Notified	Time Tissue at Hospital Lab	Time Pathologist Present at Collection	Tissue Site	Tissue Subsite	Tissue Observations	Tissue Type Tumor (T) Normal (N)	Block Size or Weight	Gross Size of Tumour	Tissue Block Observations
TI01											
TI02											
TI03											
TI04											
TI05											
TI06											
TI07											
TI08											
TI09											
TI10											
TI11											
TI12											
TI13											
TI14											

TI = Tissue

Example Biobank Tissue Handover

Biobank Packing Slip for Biospecimens Delivered to Research Lab

To: *(insert lab address)*

From: Biobank at *(insert hospital site)*

Collection Date: _____

Study Code Number: _____

Special Notes:

SAMPLES:

Ascites (mls) _____ ☐ Check = Heparin has been added

Tissue Weight (g)_____ **of Tissue Type** _____

Tissue Weight (g)_____ **of Tissue Type** _____

Tissue Weight (g)_____ **of Tissue Type** _____

Delivered by: _____

Delivered to: _____

Ascites Processing Summary Report

Study Code Number: _____ Date of Collection: _____ Time of Collection: _____

Volume: _____ mls Date Processed: _____ Processor Name: _____

Color: _____ Gross appearance: clear cloudy clots other: _____

Processing (visual): cells settling on bench top without centrifugation yes □ no □

After centrifugation: pellet color: _____ pellet size: _____

pellet consistency: _____

Supernatant Freezing:

Tube sizes: _____

-80C Freezer storage location: _____

Database updated: □

Additional processing steps (comments):

ACK: _____

Post-processing (microscopic):

Cell count: _____cells/ml = _____ Total Viability: _____

Method: trypan blue □

Cell rafts: absent □ low □ medium □ high □ RBC[#]: absent □ low □ medium □ high □

Ascites Cells Freezing:

Freeze-down vessel: Mr. Frosty □ Styrofoam box □ Other □ : _____

Freeze-down time: _____ Transfer to storage date and time: _____

Vials frozen:

# of Vials @ #cells/vial and Tube Size

LN2 Storage Location:

Freezer	Cane/ Shelf	Box	Spaces	**Database Updated?**
				□
				□
				□
				□

Tumor Sample Processing Summary Report

Study Code Number: _____ Date of Collection: _____ Time of Collection: _____

Date Received: _____ Processor Name: _____

Origin (e.g. ovary, omentum): _____

Freeze-down vessel: Mr. Frosty □ Styrofoam box □ Other □: _____

Freeze-down time: _____ Transfer to storage date and time: _____

# of tumor bulk cell vials: _____ LN2 Storage Location: _____ Database Updated: □

Metabolomic Analysis Demonstration

Background

Here, we use the R package `limma` to conduct tests for differential abundance of metabolites across sampled conditions. `limma` is flexible enough that we can specify complex models if needed, including accounting for the fact that we have different sample types that were taken from the same case (i.e., they are not independent).

We require the R packages `MetaboAnalystR` (for data pre-processing and normalization; install instructions here, including the dependency OptiLCMS.), and `limma` (for hypothesis testing). `dplyr` and `stringr` are also used for data manipulation.

```
# analysis
library(MetaboAnalystR)
library(limma)
library(dplyr)
library(stringr)
```

Reading and normalizing data

We begin by normalizing metabolite abundances using `MetaboAnalystR`. This requires that metabolite data be in a specific format; please see their website for formatting instructions, or look at the example data we have included. Alternatively, normalization can be conducted by dividing each ion count by the total ion countfor a sample, log transforming these values, then (optionally) scaling across each metabolite.

In this dataset we've previously imputed all missing values with an ion count of 1000. However, we include a minimum imputation step by MetaboAnalyst (which has no effect here). Alternatively, missing data could be imputed using other methods that attempt to supply a more informative value.

We normalize raw ion counts using a sum normalization for each sample ('SumNorm'), then a log transformation ('LogNorm'). In our manuscript we autoscaled each metabolite ('AutoNorm') afterwards, but here we illustrate skipping this step in order to use limma-trend. The results are more-or-less the same, in this case.

```
# initialize object and normalize
mSet <- MetaboAnalystR::InitDataObjects("pktable", "stat", FALSE)
mSet <- MetaboAnalystR::Read.TextData(mSet,
        '~/Documents/R/mna_ovarian/data/20190707_Cell_Screening_1213_metabo_2_clean.csv',
                        format='colu', 'disc')
mSet <- MetaboAnalystR::SanityCheckData(mSet)
mSet <- MetaboAnalystR::ReplaceMin(mSet) # set at 1000 - has no effect
mSet <- MetaboAnalystR::PreparePrenormData(mSet)
# omit scaling by setting scaleNorm = ""
mSet <- MetaboAnalystR::Normalization(mSet,
                                      rowNorm = "SumNorm",
                                      transNorm = "LogNorm",
                                      scaleNorm = ""
                                      )
```

Analysis of differential abundance

Because we wish to use a more complex statistical framework than `MetaboAnalystR` provides (or provided at the time of our study), we extract the matrix of normalized metabolite abundances from the MetaboAnalyst for downstream analysis.

```
metabo_norm <- mSet$dataSet$norm
```

Next, we need the sample metadata to construct a design matrix for statistical modelling. In this case, all of the data we need is embedded in the sample names that were formatted by MetaboAnalyst. We'll abstract these into a new `data.frame`.

We'll also make a vector corresponding to our 'condition' grouping. Although we have a two-way (factorial) design (i.e., 3 types of cells from each of 2 types of tumor compartment (ascites and tumor)), it can be difficult to interpret such models in multivariate analyses. Instead, we'll create a single grouping factor that combines cell type and tissue compartment, and test for differences between each group (see limma user's guide).

```
design_df <- metabo_norm %>%
  rownames() %>%
  str_replace('-', 'n') %>%
  str_replace('\\+', '') %>%
  str_split_fixed('_', 4) %>%
  data.frame() %>%
  `colnames<-`(c('patient', 'compartment',
                 'cell_type', 'replicate')) %>%
  mutate(group=paste(compartment, cell_type, sep='_'),
         merge_col=paste(patient, compartment, cell_type, sep='_')
  )
```

Because our data contain technical replicates for each patient by condition grouping, we'll use the average metabolite value for each grouping per patient. 'Limma can average these for us as part of the analysis. We first create an expression set for convenience.

```
# Create expression set
metabo_e <- new("EList", list(E = t(metabo_norm),
                              targets = design_df))

# Average values by patient and treatment
metabo_ave <- limma::avearrays(x = metabo_e,
                               ID = metabo_e$targets$merge_col)

# Construct matrix of dummy variables (design matrix) from treatment.
# We will also simplify the cumbersome column names of the matrix
design_matrix <- model.matrix(~ 0 + metabo_ave$targets$group)
colnames(design_matrix) <- gsub('metabo_ave\\$targets\\$group', '',
                                colnames(design_matrix))

# Make a 'block' of patient to account for correlation
# among samples from the same patient
corfit <- limma::duplicateCorrelation(object = metabo_ave,
                                      design = design_matrix,
                                      block = metabo_ave$targets$patient)
```

```
# Fit model using empirical Bayes
fit_lm <- limma::eBayes(limma::lmFit(object = metabo_ave,
                     design = design_matrix,
                     block = metabo_ave$targets$patient,
                     correlation=corfit$consensus),
                     trend = TRUE)

# We can specify the contrasts that we are interested in.
# In this case we will do all single pairwise comparisons
contr <- makeContrasts('T_8-A_8',
                       'T_4-A_4',
                       'T_45n-A_45n',
                       'T_8-T_4',
                       'T_8-T_45n',
                       'T_4-T_45n',
                       'A_8-A_4',
                       'A_8-A_45n',
                       'A_4-A_45n',
                       levels=design_matrix
)

# run model
fit_contrast <- limma::contrasts.fit(fit_lm, contrasts = contr)

# here we use limma trend to better model mean-variance relationships
eb_contrasts <- limma::eBayes(fit_contrast, trend = TRUE)
```

Now that we have fit the core model to identify differential abundant metabolites, we only need to summarize the outputs.

We can use the `decideTests` function to identify which contrasts (i.e. differences between groups) are significant, and get the most overall significantly changing metabolites using `topTable`. Here we just show a few of these for space.

```
# identify significant contrasts with decideTests
knitr::kable(limma::decideTests(eb_contrasts)[1:3, 1:5])
```

	T_8-A_8	T_4-A_4	T_45n-A_45n	T_8-T_4	T_8-T_45n
1-Methyladenosine	0	0	0	0	0
1-Methylhistidine	0	0	0	0	0
1-Methylnicotinamide	0	1	0	0	0

```
# look at most differential metabolites - just report P-values and F-stat for simplicity
knitr::kable(limma::topTable(eb_contrasts, n=5, coef=1:9)[, 11:13])
```

	F	P.Value	adj.P.Val
1-Methylnicotinamide	23.970368	0.0000000	0.0000003
Xanthine	8.085605	0.0000813	0.0040242
Xanthosine	6.944117	0.0002518	0.0065067
L-Alanine	6.902235	0.0002629	0.0065067

	F	P.Value	adj.P.Val
Deoxycytidine	6.217229	0.0005422	0.0107364

##Plotting

Custom plot or further analyses (e.g. PCA, etc) can be conducted from the normalized data matrix and/or the limma outputs. Keep in mind that the metabolites have not yet been scaled, so scaling may desirable for some outputs.

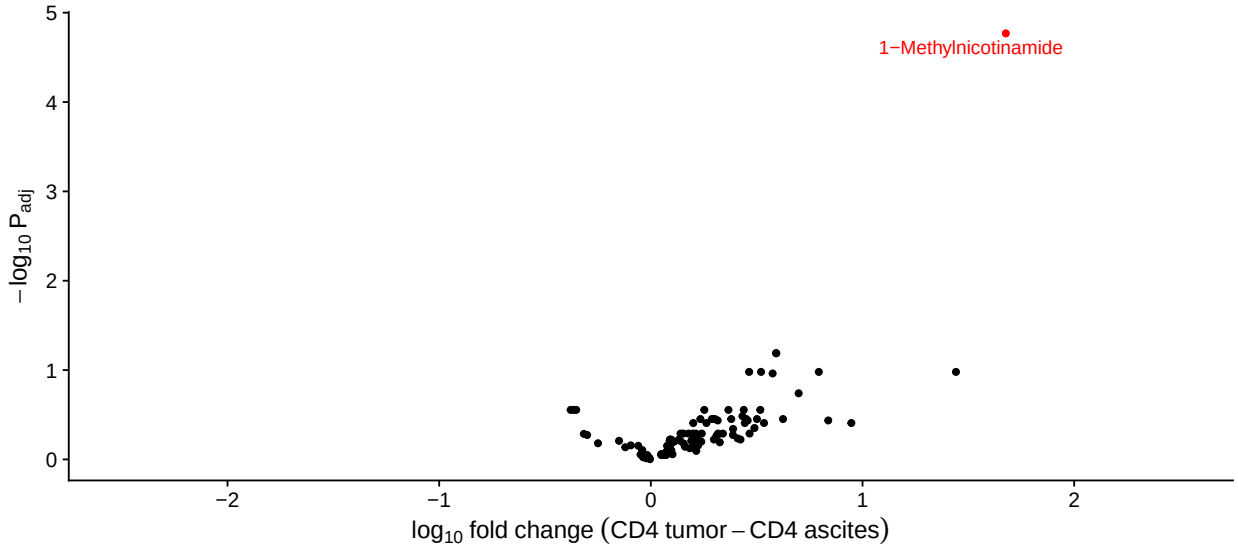
Below we show simple volcano plot for differentially abundant metabolites between the tumor and ascites compartments for CD8 and CD4 t cells.

```
# we can make volcano plots based on the topTable output
# we use ggplot here
library(ggplot2)
library(ggrepel)
library(cowplot)


# we need to call topTable for the specific contrast we are interested
# in to get the appropropriate P values
# add a column of significant names for labeling
tt <- limma::topTable(eb_contrasts, n=Inf, coef=2)
tt$rn <- ifelse(tt$adj.P.Val < 0.05, rownames(tt), '')

# plots - here

ggplot(tt, aes(x=logFC, y=-log10(adj.P.Val),
               color=ifelse(adj.P.Val < 0.05, 's', 'n.s') )) +
  geom_point() + xlim(c(-2.5, 2.5)) +
  guides(color='none') +
  scale_color_manual('', values=c('black', 'red')) +
  geom_text_repel(label=tt$rn) +
  ylab(expression(-log[10]~P[adj])) +
  xlab(expression(log[10]~fold~change~(CD4~tumor-CD4~ascites))) +
  theme_cowplot()
```


1−Methylnicotinamide
5
4
3
2
1
0
−log10 Padj
−2
−1
0
1
2
log10 fold change (CD4 tumor − CD4 ascites)