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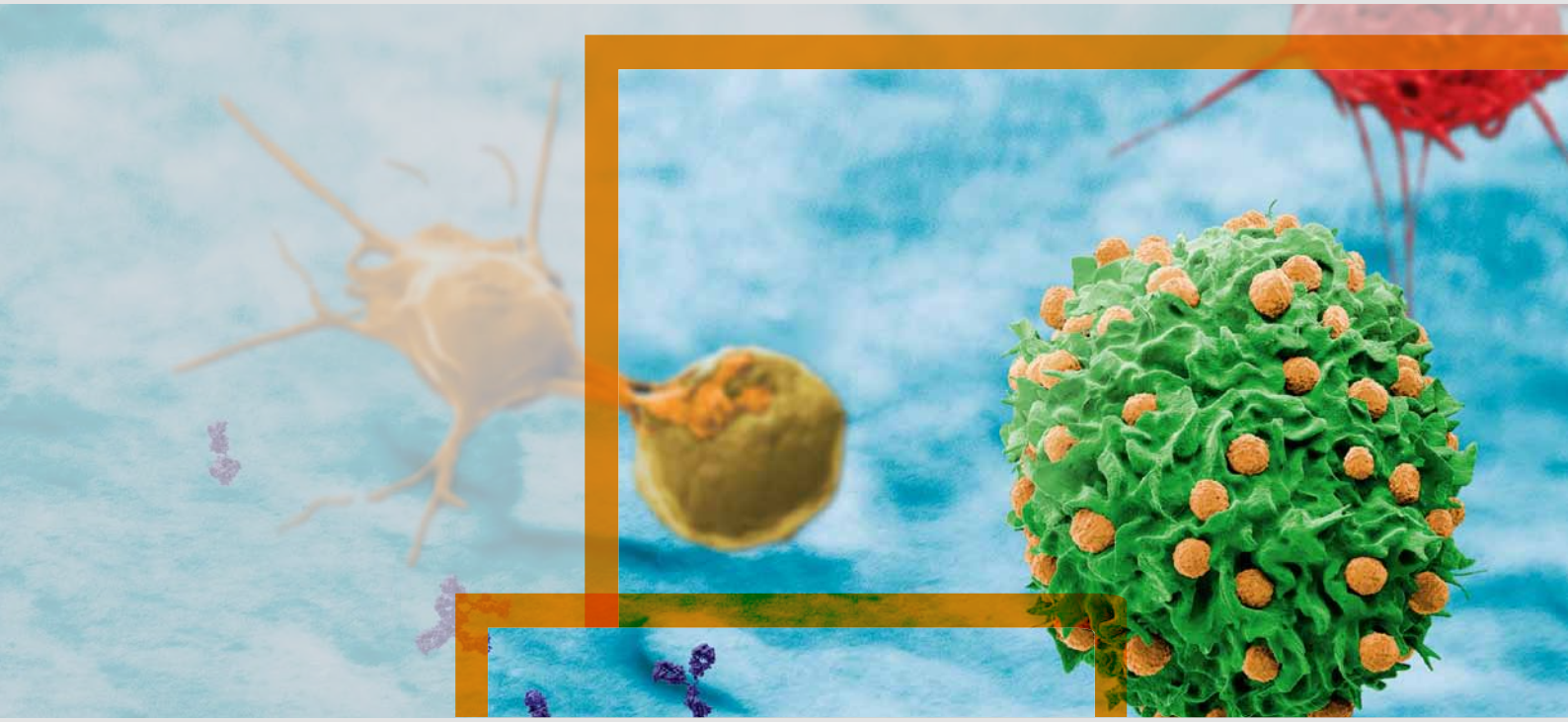

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# Isolation of monocytes with high purity directly from whole blood for transcriptome analysis in translational research

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## Introduction

Fenofibrate is a lipid-lowering drug used in the treatment of dyslipidemia. Fenofibrate effects have been attributed to the activation of the nuclear transcription factor peroxisome proliferator–activated receptor-α (PPARα). PPARα plays a role in the regulation of tissue factor expression in human monocytes and might thus influence atherothrombosis[1,2].

In this study, we assessed the effects of fenofibrate on gene expression in peripheral blood monocytes of healthy donors. Microarray-based transcriptome analysis is a powerful tool in translational research for evaluating drug effects on particular blood cell types. However, for the reliable detection of subtle changes in gene expression in a certain cell type, it is crucial to use isolated cell populations of high purity for analysis.

In particular for translational research projects involving multiple clinical centers and operators, it is vital to use cell isolation protocols that are short, simple, and highly reproducible, and avoid steps that are prone to variability, such as the preparation of peripheral blood mononuclear cells (PBMCs). Positive selection of CD14$^+$ monocytes by MACS® Technology is a well-established and reliable procedure. Here we used the autoMACS® Pro Separator and Whole Blood CD14 MicroBeads to magnetically isolate monocytes directly from whole blood and achieved high cell purities and yields. This protocol avoids both PBMC preparation and erythrocyte lysis, which are both laborious and can lead to variation in cell separation results.

Microarray experiments and comparison of gene expression at three different time points of fenofibrate treatment allowed for the identification of differentially expressed sequences (DES) and modulated biological functions. The experiment workflow is summarized in figure 1.

## Subjects, materials, and methods

### Subjects and study design

Twenty six healthy males or post menopausal (natural or chirurgical) females not receiving hormone replacement therapy (HRT) or having stopped HRT for at least 1 month, aged 40–65 years inclusive, were recruited in this open-label, single-center research study to receive a standard dose of fenofibrate as one 145 mg tablet daily treatment (Lipanthyl®, Laboratoires Fournier SA, Dijon, France). Subjects with a body mass index (BMI) ≥30 kg/m$^2$ or <18 kg/m$^2$ or with known hypersensitivity to fibrates, or females with child bearing potential without a reliable method of contraception, having received an investigational drug in the last 90 days before date of inclusion were not included in the study. All subjects had normal folate levels (mean±sd: 15.2±11.1 ng/mL) and vitamin B12 levels (404.1±165.2 pg/mL) at inclusion in the study. The study included a screening phase from a few days up to 3 weeks (wks) and a treatment phase of 6 wks. Blood was drawn and monocytes were separated at baseline, after 7 to 10 days, and after 6 wks of treatment.

Ethic approval was obtained from the Guy's Hospital Research Ethics Committee, London, UK. Freely given informed consent was obtained from each subject before enrollment.

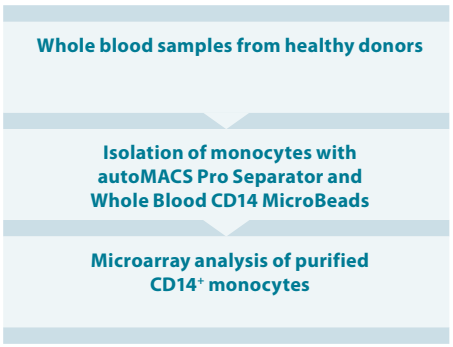



**Figure 1 Workflow for the isolation of CD14$^+$ monocytes directly from whole blood and subsequent transcriptome analysis.**

### Isolation of monocytes from whole blood

Freshly drawn whole blood (40 mL) was anticoagulated using EDTA. Two aliquots (15 mL each) were magnetically labeled with Whole Blood CD14 MicroBeads. Labeled CD14$^+$ monocytes were automatically isolated in two runs using the autoMACS Pro Separator according to the manufacturer's protocol. Isolated monocytes from both runs were combined and analyzed by flow cytometry to determine viability, yield, and purity. For subsequent RNA extraction, isolated cells were centrifuged and flash-frozen.

### Flow cytometry

Cells were labeled with CD14-PE and CD45-FITC antibodies before and after separation, and analyzed by flow cytometry. CD15 antibodies were used to evaluate the frequency of granulocytes. All antibodies were obtained from Miltenyi Biotec. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.

### RNA extraction and microarray analysis

Total RNA was extracted from flash-frozen cell samples using the NucleoSpin® RNA II system (Macherey-Nagel). RNA was quantitated and RIN values were assessed using the Agilent 2100 Bioanalyzer platform and the integrated software. RNA was amplified and labeled with Cy™3 using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies). RNA was hybridized to Agilent Whole Human Genome Oligo Microarrays (4×44K).

### Statistical analysis

A global effect of the treatment on the 78 (26×3) intensity profiles was determined using a 2-way ANOVA considering donors and times of sample collection, adjusted by using the Benjamini-Hochberg false-discovery rate (FDR) method to adjust $p$ values and control for the first species error. Pairwise comparisons of the DES between study visits were performed using the Student-Newman-Keuls (NK) test. A significant effect of the treatment on the sequences was concluded for $p \leq 0.01$. For functional analysis, pathways and networks were constructed based on the classification of all the DES modulated in at least one pairwise comparison ($p \leq 0.01$ for NK test) via a k-means clustering approach (user-defined number of clusters = 11, cosine correlation and centroid-based search). Functional networks were constructed using the Ingenuity® Pathway Analysis software (IPA version 8.6 build 93815).

## Results and discussion

### Magnetic isolation of monocytes directly from whole blood

$CD14^+$ monocytes were enriched directly from whole blood in an automated fashion using the autoMACS Pro Separator and Whole Blood CD14 MicroBeads. In the example shown in figure 2 the frequency of monocytes in the whole blood sample amounted to ≈6%. MACS Technology allowed us to separate monocytes to purities greater than 97% (fig. 2, dot plots). The mean purity of monocytes in 78 samples from 26 donors amounted to 94.1±6.17%. The mean cell yield was $1.3\times10^6\pm0.55\times10^6$. Purified monocytes showed consistently high viabilities of ≈96%. For details see the table in figure 2.

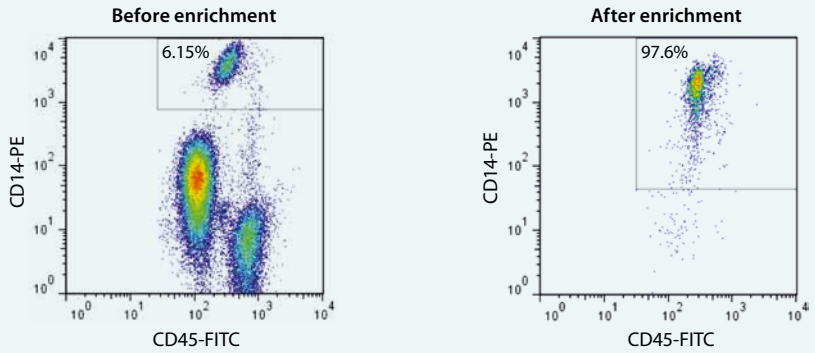
### Quality and yield of RNA extracted from isolated monocytes

The yield of monocytes magnetically isolated from 30 mL of whole blood was high, which allowed us to extract large amounts of RNA for microarray analysis. The yield of RNA extracted from isolated monocytes (n=78) amounted to 2.15±0.73 µg (mean±sd). RIN values reached 9.57±0.47 (mean±sd) indicating consistently high RNA quality for sensitive, reliable microarray experiments.

### Microarray analysis of monocytes isolated with MACS® Technology

We compared gene expression in monocytes isolated from peripheral blood of healthy donors at 0, 1, and 6 wks of fenofibrate treatment. We identified 5,187 sequences that were differentially modulated in at least one of the pairwise comparisons. The distribution was as follows: 3,924 DES between 0 and 1 wk, 1,973 DES between 0 and 6 wks, and 2,904 DES between 1 and 6 wks of fenofibrate treatment (fig. 3).

K-means clustering led to the identification of 11 clusters showing different trends over time in gene expression after fenofibrate treatment. One of the k-means clusters, which included 476 DES, showed a trend to down-regulation after 1 wk and a trend to up-regulation between 1 and 6 wks of treatment. Out of these 476 DES, 324 had mapped gene identities, 231 were eligible for networks, and 220 were eligible for function pathway analysis around immunological disease, cell-mediated immune response, cellular assembly and organization, cellular movement, antigen presentation, cardiovascular disease, cellular growth and proliferation, lipid metabolism, molecular transport, inflammatory response etc. (fig. 4).




| Parameter | Mean | sd |
|---|---|---|
| Frequency of monocytes in whole blood sample (% among $CD45^+$ cells) | 8.1 | 2.25 |
| Purity of monocytes after enrichment (% among $CD45^+$ cells) | 94.1 | 6.17 |
| Yield of enriched cells (number of cells per mL) | $1.3\times10^6$ | $0.55\times10^6$ |
| Viability of enriched cells (% live cells) | 95.7 | 3.4 |

**Figure 2 Isolation of $CD14^+$ monocytes from whole blood using the autoMACS® Pro Separator and Whole Blood CD14 MicroBeads.** Cells were enriched as indicated in the subjects, materials, and methods section. Before and after enrichment, cells were labeled with CD14-PE and CD45-FITC and analyzed by flow cytometry.

## Conclusion

- The autoMACS Pro Separator in combination with Whole Blood CD14 MicroBeads allows for rapid and robust magnetic isolation of monocytes with high yields.
- Isolated CD14$^+$ monocytes showed purities of 94% on average.
- The use of isolated monocytes enables sensitive and accurate microarray-based transcriptome analysis of research samples.
- Significant short-term (1 wk) and middle-term (6 wks) effects of fenofibrate on gene expression in monocytes were observed.
- Identification of a large number of DES within a k-means cluster allowed for the construction of functional networks with eligible gene identities involved in particular in metabolic and inflammatory pathways.

**References**

1. Marx, N. *et al.* (2001) Circulation 103: 213–219.
2. Neve, B.P. *et al.* (2001) Circulation 103: 207–212.

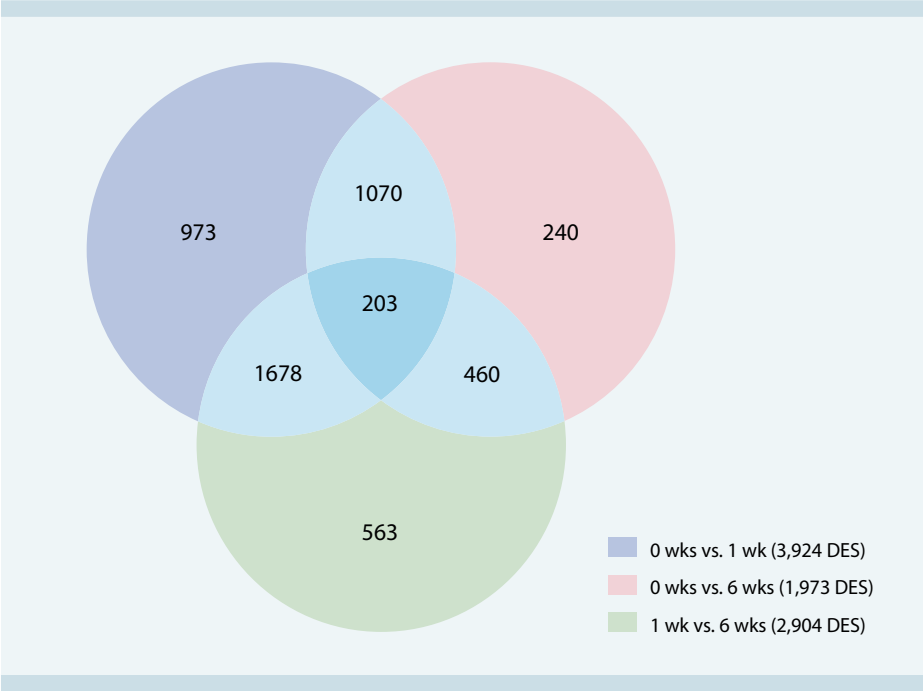



**Figure 3 Venn diagram for comparisons of DES at three time points of fenofibrate treatment.** The Student-Newman-Keuls algorithm was applied for all pairwise comparisons. For details see the subjects, materials, and methods section.

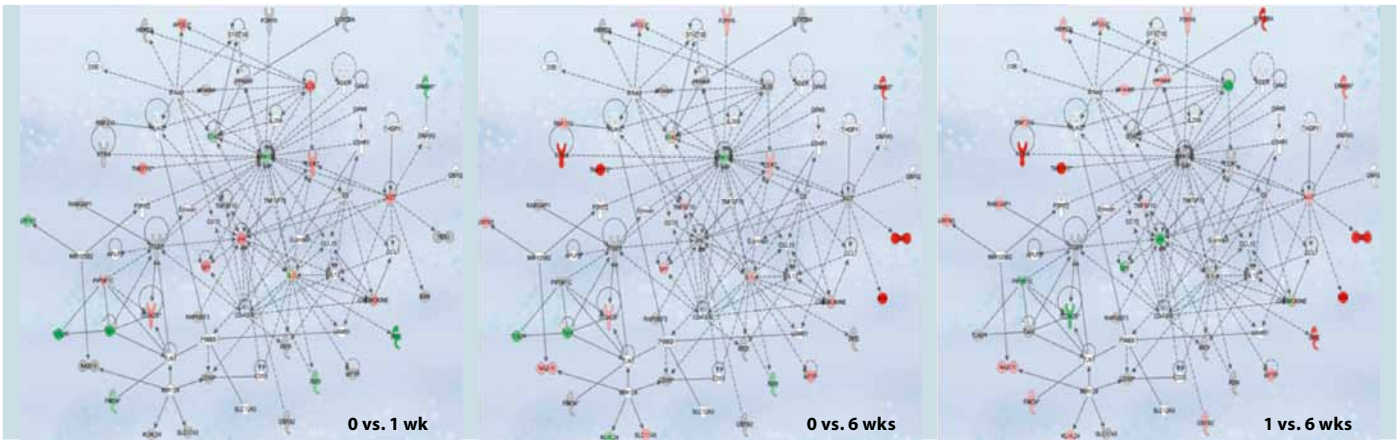



**Figure 4 Functional networks constructed from a k-means cluster.** Network-eligible genes from a cluster showing a trend to down-regulation after 1 wk and a trend to up-regulation between 1 and 6 wks of fenofibrate treatment were used as "seeds" for *in silico* network generation. Down-regulation is indicated in green and up-regulation in red. For details see the subjects, materials, and methods section.

| MACS Product or Services* | Order no. |
|---|---|
| autoMACS Pro Separator – Starter Kit | 130-092-545 |
| Whole Blood CD14 MicroBeads, human | 130-090-879 |
| CD14, CD15, and CD45 antibodies | Visit www.macsantibodies.com |
| Genomic services | Visit www.macsgenomicservices.com |

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