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DIRETORIA DE TRATAMENTO DA INFORMAÇÃO

Cidade Universitária Zeferino Vaz Barão Geraldo

CEP 13083-970 – Campinas SP

Fone: (19) 3521-6493

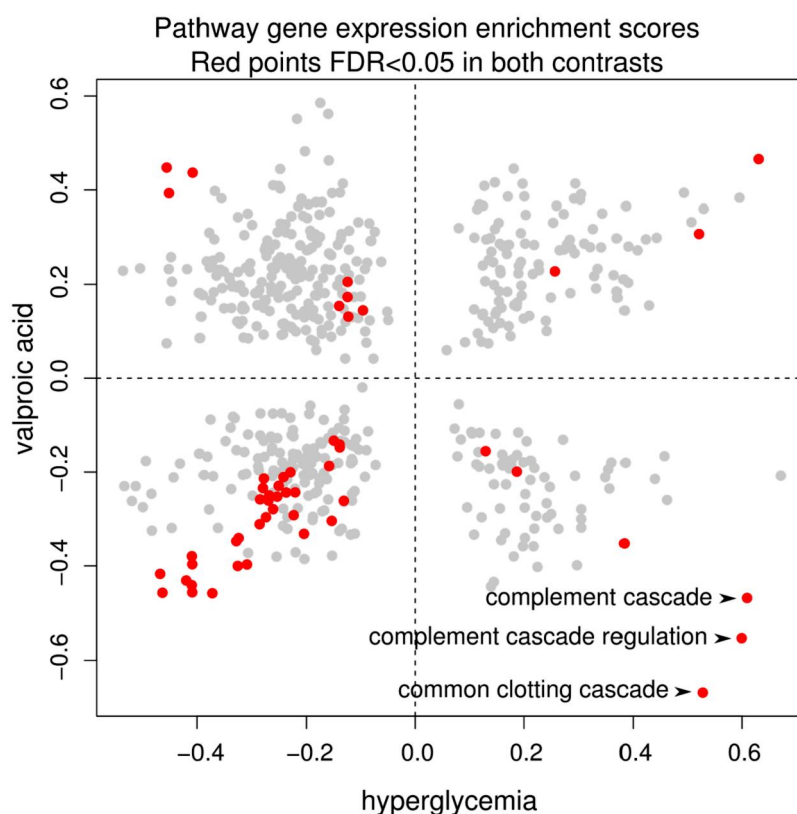
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ABSTRACT

Atherothrombosis remains the leading cause of morbidity and mortality in patients diagnosed with diabetes mellitus, but the molecular mechanisms underpinning this remain unresolved. As the liver plays a major role in metabolic homeostasis and secretion of clotting factors and inflammatory innate immune proteins, there is an interest in understanding the mechanisms of hepatic cell activation under hyperglycemia and whether this can be attenuated pharmacologically. We have previously shown that hyperglycemia stimulates major changes in chromatin organisation and metabolism in hepatocytes, and that the histone deacetylase inhibitor valproic acid (VPA; IUPAC: 2-propylpentanoic acid) is able to reverse some of these metabolic changes. In this study, we used deep transcriptome sequencing to show that VPA attenuates hyperglycemia-induced activation of complement and coagulation cascade genes. These findings reveal a novel mechanism of VPA protection against hyperglycemia, which might improve the therapeutic approaches for diabetes.

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Hyperglycemia induces an inflammatory, prothrombotic state in diabetic patients but the underlying mechanisms remain poorly understood. Felisbino et al, use transcriptomics to identify molecular pathways upregulated by hyperglycemia in hepatocytes, and show that valproic acid attenuates the activation of coagulation and complement genes, which may be useful to reduce cardiovascular risk in diabetes patients.



INTRODUCTION

Diabetes is a multifactorial disorder with several pathways implicated in the development of diabetic micro- and macro-vascular complications.^[1] Macrovascular complications include atherosclerosis, cerebrovascular disease and peripheral vascular disease; for which diabetic patients have a two- to four-fold greater risk than non-diabetic individuals.^[2] In diabetes, a combination of hyperglycemia, inflammation, oxidative stress and insulin resistance converge to produce a prothrombotic milieu, characterised by endothelial dysfunction, coagulative activation and platelet hyperreactivity.^[3] Several important coagulation pathway proteins are elevated by hyperglycemia *in vivo* including fibrinogen, prothrombin 1 and 2, tissue factor, thrombin-antithrombin complexes, plasminogen activator inhibitor-1, tissue plasminogen activator and complement C3 which contribute to this hypercoagulative state.^[4,5] This prothrombotic state appears to occur simultaneously with and may be dependent upon chronic low-grade inflammation and oxidative stress observed in diabetes.^[6] Complement cascade proteins are a proposed source of inflammation in diabetes that are produced by monocytes, macrophages and hepatocytes. These proteins function in innate antimicrobial defense primarily through membrane attack and phagocyte recruitment, that are elevated in diabetes and suspected to contribute to diabetic complications.^[7] The liver is the main site of production of circulating coagulation and complement proteins, and responsible for production of bile, cholesterol, decomposition of red blood cells and detoxification of xenobiotics and secondary metabolites. Of key importance for diabetes, liver hepatocytes play a major role in energy homeostasis by storing carbohydrates as glycogen during hyperglycemia and releasing

sugars during hypoglycemia. Liver dysfunction, classified as elevated hepatic glucose production during hyperglycemia, is common in type-2 diabetes, and inhibiting this is a major mechanism of action of the widely prescribed glucose lowering drug metformin.^[8] Thus, understanding the molecular mechanisms underpinning the hyperglycemic activation of metabolism, coagulation, complement and other inflammatory pathways in hepatocytes could identify new therapies to reduce the burden of diabetic complications.

At the interface between genetic and environmental factors, epigenetic mechanisms are proposed to play a major role in the development of metabolic disease including diabetic complications.^[9,10] Previous reports have demonstrated that chromatin remodeling and histone acetylation are important mechanisms in diabetes development.^[11,12] The epigenetic component of metabolic/inflammatory disorders has come recently to attention, revealing epigenetic drugs as potential immunomodulatory agents. The recent discovery that histone deacetylase (HDAC) inhibitors (HDACi) have the ability to reduce the severity of inflammatory and autoimmune diseases, including diabetes, in several animal models, has positioned them as alternative anti-inflammatory agents.^[13] Their paradigmatic mode of action has been defined as increased histone acetylation of target genes, leading to higher gene expression; however, recent studies have shown a more diverse mechanism of gene regulation.^[14-17]

Valproic acid (VPA), the most clinically prescribed HDACi, is a fatty acid with anticonvulsant properties used for the treatment of epilepsy and seizures.^[18] Recently, it has been investigated in different disease states as part of a strategy to repurpose clinically approved drugs.^[19-21] There are reports of VPA reducing the blood glucose level and fat deposition in

adipose tissue and liver,^[22,23] as well as controlling the insulin production ^[24] and gluconeogenesis signaling.^[23,25,26] It also seems to improve the microvascular complications of diabetes.^[27,28]

We have previously shown that treatment of HepG2 human hepatocytes with the HDACis Trichostatin A (TSA) and VPA attenuated hepatic glucose production, although no significant difference was detected in global chromatin structure and epigenetic landscape. Chromatin alterations promoted by HDACi under hyperglycemia may be a function of the differently regulated nuclear domains and genes rather than of global remodeling.^[12]

Therefore, identification of genes impacted by HDAC inhibition is paramount to a comprehensive understanding of its mechanisms of action and therapeutic target in amelioration of hyperglycemic state.^[15]

We hypothesise that hepatocytes undergo major gene expression alterations when exposed to a hyperglycemic environment as the liver is an organ of critical importance to carbohydrate metabolism. Furthermore, we hypothesised that VPA could attenuate some of the deleterious pathways promoted by hyperglycemia.

In this study, HepG2 cells exposed to high-glucose (HG) were stimulated with VPA. We performed high throughput RNA-sequencing (RNA-seq) to understand transcriptome-wide analysis of genes and pathways in response to hyperglycemia and VPA. This work identified that complement and coagulation pathways activated by hyperglycemia were strongly attenuated by HDAC inhibition. This work suggests a new avenue of action of VPA that might be relevant to the management of diabetes.

RESULTS

Hyperglycemia severely impacts hepatocyte gene expression

In order to understand the effect of high glucose on whole genome hepatic gene expression and function, RNA-seq was performed in HepG2 cells stimulated by hyperglycemic conditions in triplicate. After read alignment and gene expression quantification, statistical analysis of genes and pathways was undertaken. Multidimensional scaling analysis measures the similarity of the samples and projects this on two-dimensions. We observe low glucose (LG) and high glucose (HG) samples clustered into distinct groups (Figure 1A). Statistical analysis showed that HG treatment had a strong effect on HepG2 cells, with 4,259 genes showing differential expression ($FDR \leq 0.05$; Figure 1B – red points). This effect is much higher than that reported for high glucose treated monocytes ^[29] and endothelial cells ^[30] suggesting that hepatic cells are sensitive to changes in carbohydrate supply.

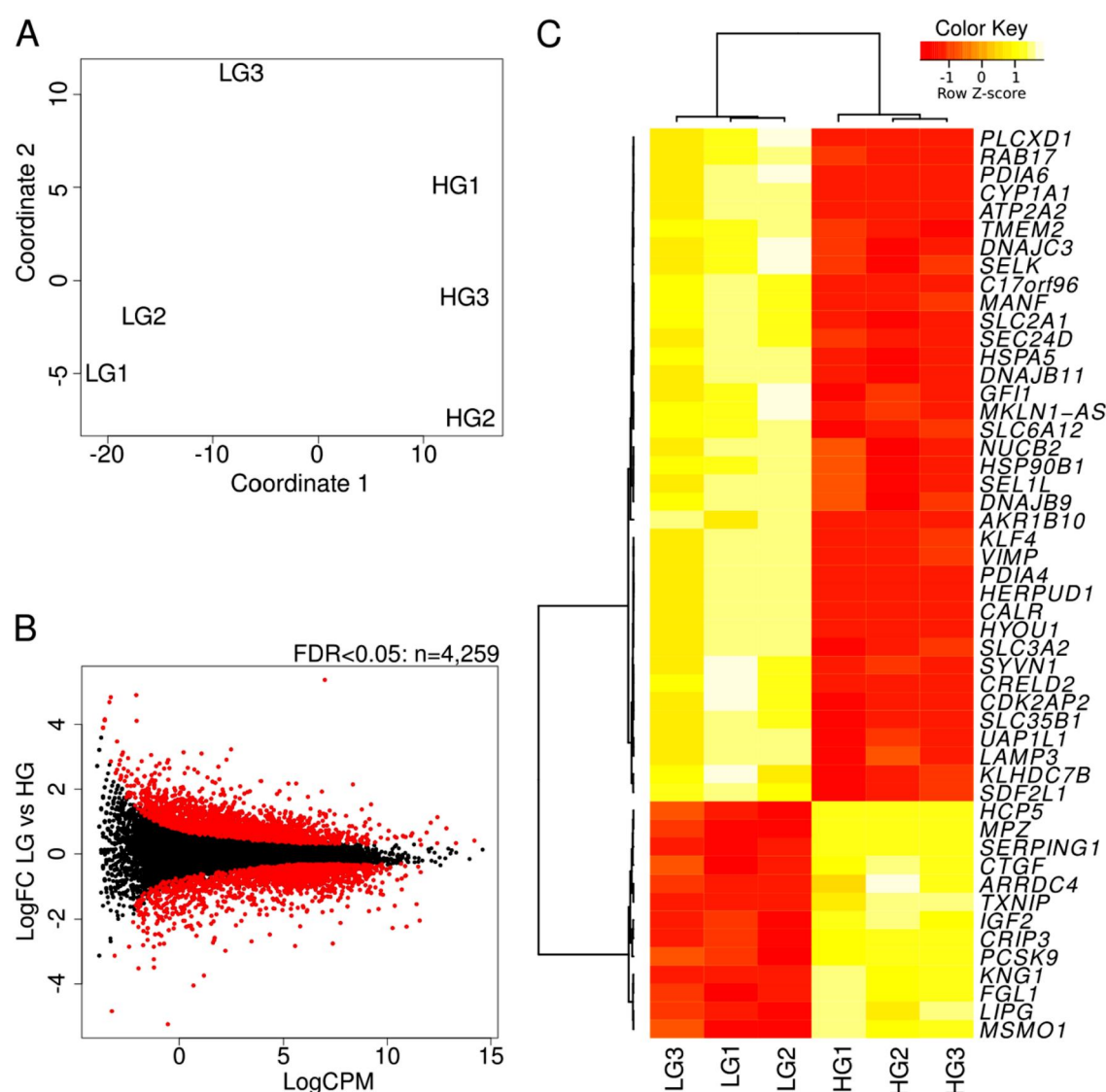


Figure 1

Figure 1. HepG2 gene expression changes in response to high glucose by RNA-seq analysis. (A) Multidimensional scaling analysis of library size normalized counts shows sample clusters based upon treatment on dimension 1. (B) Smear plot depicts the log2 fold change and average expression level log2 counts per million for each detected gene. Genes with differential expression (FDR≤0.05) are highlighted in red. (C) Heatmap of the 50 most significant differentially expressed genes. LG, low glucose – normoglycemic condition. HG, high glucose – hyperglycemic condition.

The top 50 differentially expressed genes by significance are shown in heatmap form (Figure 1C). Some of the up-regulated genes are: *TXNIP*, a known hyperglycemia inducible gene that is highly abundant in HepG2 cells (TXNIP protein inhibits the normal function of thioredoxin leading to accumulation of ROS); *SERPING1*, which encodes C1 esterase inhibitor, and is involved in inhibition of complement cascade; *MPZ*, that encodes a structural component of the myelin sheath and is thought to be specific to nervous system tissues; *MSMO1*, that encodes a protein involved in cholesterol biosynthesis; *PCSK9*, whose protein acts in binding to and degrading low-density lipid receptors; *IGF2*, that encodes an insulin-like growth factor, the central regulator of somatic growth and cell proliferation. Some of the down-regulated genes are: *MANF*, whose protein promotes survival of dopaminergic neurons, possibly playing a role in ER stress response; *HSPA5* (aka *GRP78*), which encodes a glucose sensing protein, whose expression is upregulated by glucose starvation and is also present in the ER; *CALR*, that encodes calreticulin, a major calcium storage protein in the ER; *DNAJB11*, which encodes yet another ER localized protein that acts as a chaperone for a number of partners. The glucose transporter gene *SLC2A1*, encoding GLUT1 was also strongly down-regulated.

Gene Set Enrichment Analysis (GSEA) was used in order to understand pathways regulated by hyperglycemia. From 575 REACTOME gene sets considered, 34 were upregulated and 139 were down-regulated ($FDR \leq 0.05$). The top 20 gene sets by significance in the up and down-regulated directions are shown (Figure 2A). Down-regulated gene sets included those associated with extracellular matrix interactions, chaperone function, calnexin/calreticulin cycle,

N-glycan trimming and peptide chain elongation (Figure 2A), while gene sets upregulated in response to hyperglycemia included cholesterol biosynthesis, complement cascade and fibrin clotting cascade (Figure 2B, C, D). These findings show a distinctive response of hepatocytes to hyperglycemia.

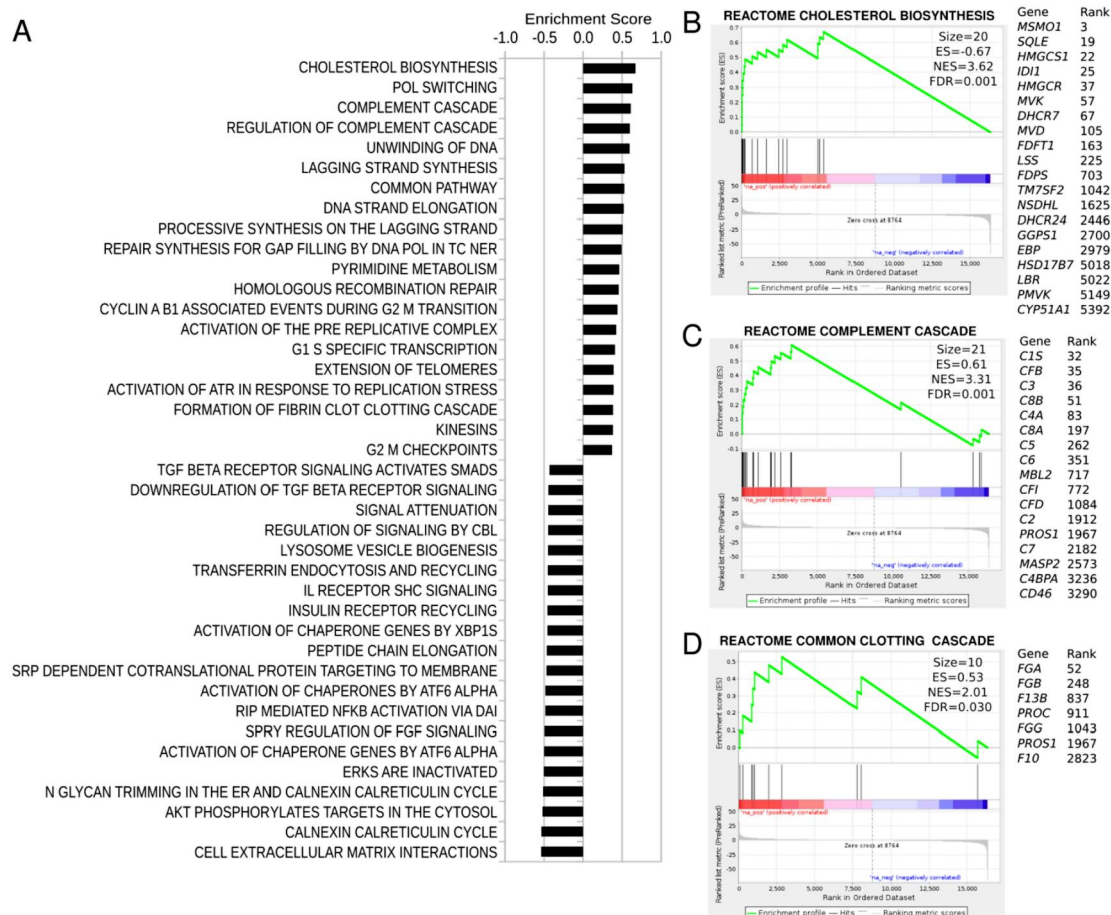


Figure 2

Figure 2. Gene sets differentially expressed in response to high glucose. (A) Top 20 REACTOME pathways with differential expression selected by statistical significance, determined by pre ranked gene set enrichment analysis (GSEA-P). (B, C, D) Enrichment plots show upregulation of cholesterol biosynthesis, complement cascade and common clotting cascade in response to hyperglycemia. All pathways GSEA FDR<0.05.

VPA treatment attenuates the expression of hyperglycemic response genes

Given that hyperglycemia induces major changes to the hepatocyte transcriptome and activates pathways relevant to cardiovascular health (such as cholesterol metabolism and complement/clotting cascades) and our previous work shows that VPA attenuates hepatic function, we hypothesised that VPA might inhibit hyperglycemic gene expression signatures.

Multidimensional scaling analysis shows that samples cluster based on treatment group. Untreated samples (LG, HG) are clearly separated from VPA-treated samples (LGV, HGV); and normoglycemic samples (LG, LGV) are separated from hyperglycemic ones (HG, HGV) (Figure 3A). Smear plot shows that 7,802 genes were altered in expression due to VPA treatment under hyperglycemia (Figure 3B). This plot also shows genes with initially low expression were upregulated after VPA treatment; on the other hand, genes initially highly expressed were down-regulated. Heatmap of top 50 genes by significance shows that the majority were upregulated (Figure 3C).

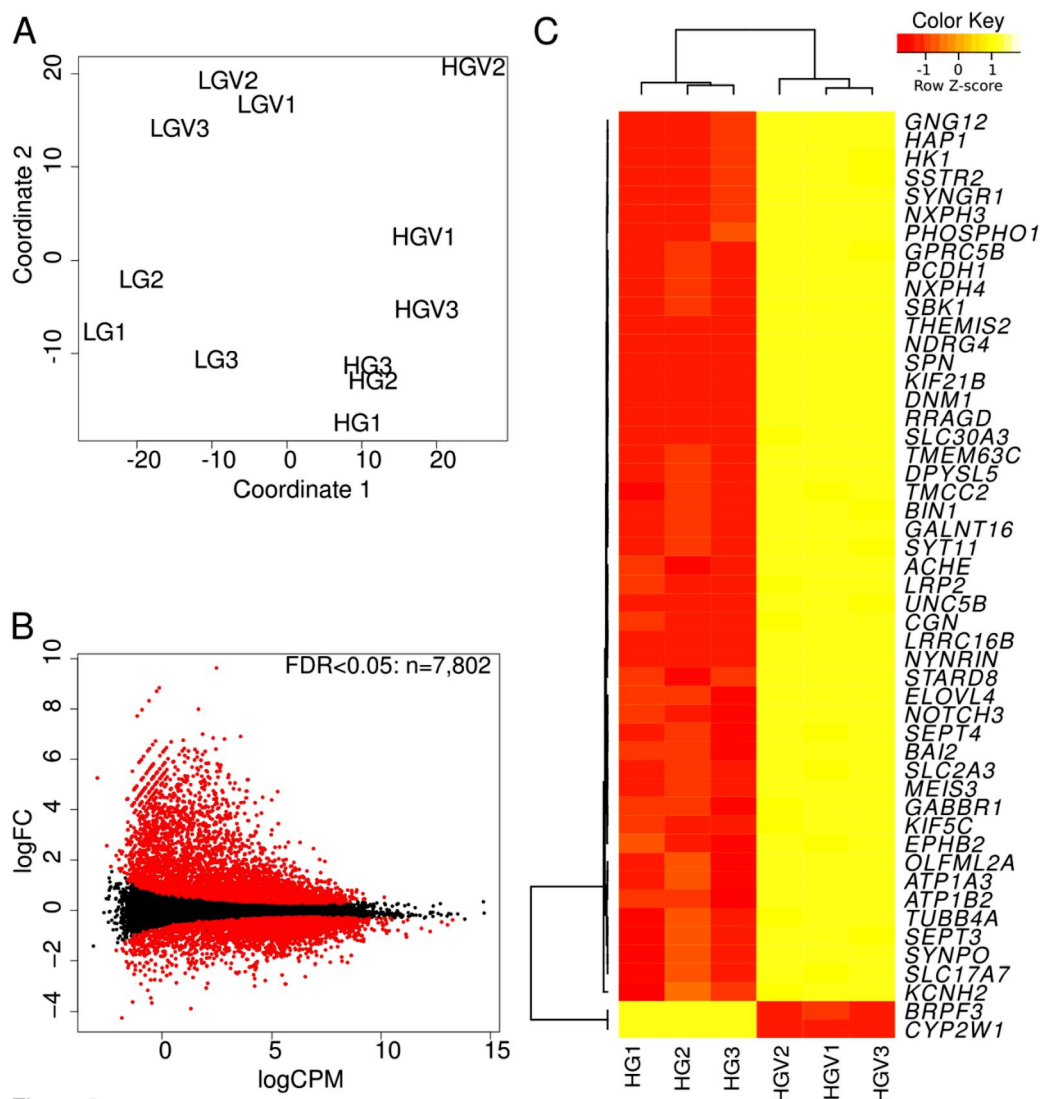


Figure 3

Figure 3. Hyperglycemic HepG2 gene expression in response to VPA. (A) Multidimensional scaling analysis of library size normalized counts shows samples cluster based upon treatment groups. (B) Smear plot showing the effect of VPA treatment on hyperglycemic HepG2 cells. Genes with differential expression ($FDR \leq 0.05$) are highlighted in red. (C) Heatmap of the 50 most significant differentially expressed genes responding to VPA. LG, low glucose – normoglycemic condition. LGV, normoglycemic condition followed by VPA treatment. HG, high glucose – hyperglycemic condition. HGV, hyperglycemic condition followed by VPA treatment

The top 20 gene sets by significance in the up- and down-regulated directions are shown (Figure 4A). Gene sets upregulated included those related to function of neurons including potassium channels, neurotransmitter receptor, L1-type/ankyrins interactions. Down-regulated gene sets included common pathway of fibrin clot formation, complement cascade and genes involved in protein synthesis. Clotting and complement cascade genes were down-regulated by VPA in hyperglycemic condition (Figure 4B,C). The regulation of all genes in response to glucose and VPA was visualised on a two dimensional rank-rank plot (Figure 4D). We observe that overall, genes are distributed relatively evenly among the four quadrants.

Using rank-rank visualisation of clotting and complement cascade genes we observed coordinated upregulation of these genes with hyperglycemia and attenuation by VPA (Figure 4E,F). The FDR corrected MANOVA p-values for the two-dimensional association were 2.0E-4 and 1.5E-7 for clotting and complement cascades respectively.