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A connectivity mapping approach predicted acetylsalicylic acid (aspirin) to induce osteo/odontogenic differentiation of dental pulp cells

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Abstract

Aim To use the connectivity map, a bioinformatics approach, to identify compounds that could induce odontogenic differentiation of dental pulp cells (DPCs) and to experimentally validate this effect. A subsidiary aim was to investigate the anti-inflammatory effect of any identified compound.

Methodology The Gene Expression Omnibus (GEO) database was searched for microarray datasets assessing odontogenic differentiation of human DPCs. An odontogenic gene expression signature was generated by differential expression analysis. The statistical significant connectivity map (ssCMap) method was used to identify compounds with a highly correlating gene expression pattern. DPCs were treated with the compound identified and osteo/odontogenic differentiation was assessed by alizarin red staining, alkaline phosphatase activity and expression of osteo/odontogenic genes ALPL, RUNX2, COL1A1, DSPP, DMP1 and SPP1 by RT-PCR. The anti-inflammatory effect of the compound was assessed using an *ex vivo* pulpitis model and cytokine levels were measured with multiplex assay. Means were compared using the *t- test or ANOVA* followed by a Bonferroni post hoc -test with the level of significance set at $p \leq 0.05$.

Results The GEO database search identified a specific gene expression signature for osteo/odontogenic differentiation. Analysis using the ssCMap found that acetylsalicylic acid (ASA)/aspirin was the drug with the strongest correlation to that gene signature. The treatment of DPCs with 0.05 mM ASA showed increased alkaline phosphatase activity ($p<0.001$), mineralisation ($p<0.05$), and increased the expression of the osteo/odontogenic genes, DMP1 and DSPP ($p<0.05$). Low concentration (0.05mM) ASA reduced inflammatory cytokines IL-6 ($p<0.001$), CCL21 ($P<0.05$) and MMP-9 ($p<0.05$) in an *ex vivo* pulpitis model.

Conclusions Connectivity mapping, a web based informatics method, was successfully used to identify aspirin as a candidate drug that could modulate the differentiation of DPCs. Aspirin was shown to induce odontogenic differentiation in DPCs *in vitro* and this, together with its anti-inflammatory effects, makes it a potential candidate for vital pulp therapies.

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Introduction

The dental pulp is populated with multipotent adult stem cells that confer capacity to regenerate the dentine-pulp complex following damage caused by caries and trauma (Sloan & Smith 2007). Vital pulp therapies including direct and indirect pulp capping offer opportunities for repair provided an optimal environment is created for activation, recruitment and differentiation of dental pulp stem cells into odontoblast-like cells to produce tertiary dentine. Traditionally, calcium hydroxide has been the material of choice for pulp capping but clinical long term outcomes have been controversial (Murray & García-Godoy 2006). The introduction of calcium silicate cements has resulted in improved clinical outcomes, compared to calcium hydroxide (Li *et al*. 2015), but there is still an unmet need for biologically based therapies for dentine repair and regeneration (Kearney *et al*. 2018).

The improved understanding of the biological processes associated with pulp-dentine regeneration facilitated and informed research leading to the discovery of biologically based pulp therapies. In this regard the use of bioactive molecules, such as TGF-β, was shown to enhance the regenerative capacity of stem cells and subsequent dentine regeneration (Goldberg *et al*. 2001, Shrestha & Kishen 2017). Other approaches that utilised biomolecules known to induce osteogenic effects, demonstrated successful odontogenic differentiation of dental pulp cells (DPCs) (Phung *et al*. 2017). In addition pharmacological targeting of the Wnt/β catenin signalling pathway, which is known to have a role in dentine repair, demonstrated dentine formation *in vivo* (Neves *et al*. 2017).

Bioinformatics provides a novel approach to identify compounds or molecules that could induce dentine formation, based on their biological functions. Gene expression connectivity mapping (CMap) is an innovative web-based technique to establish connections via gene expression profiles/signatures (www.broadinstitute.org/cmap/). The CMap uses a reference database of gene expression profiles representing a series of structured microarray experiments in cell lines to various compounds or drugs. The basic concept of the CMap is to interrogate the reference database with the specific gene expression signature of a disease or a biological condition. Theoretically, these genes may be important for the induction of the phenotype of interest, therefore similar alteration of gene expression by any compound or drug identified would be predicted to induce the same phenotype. One major application of connectivity mapping is to identify potential small molecules able to regulate the expression of a small number of genes (Lamb *et al.* 2006, Lamb, 2007).

CMap involves the use of a pattern-matching algorithm with a high level of resolution and specificity, however, it has limitations. The method has been further developed into the 'statistically significant connectivity map' (ssCMap) to strengthen reliability by using statistical

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means to control for false connections between gene signature and reference profiles (Zhang & Gant 2008, 2009, Musa *et al*. 2017). The ssCMap has been successfully applied to phenotypic targeting and predicting effective drugs for several diseases (Ramsey *et al*. 2013, Malcomson *et al*. 2016).

Based on phenotypic targeting, we hypothesised that connectivity mapping could be used to identify compounds or drugs that could shift undifferentiated DPCs towards an odontogenic phenotype. The aim of this study was to use publicly available gene expression datasets, together with ssCMap to identify compounds or drugs, already licensed for use in humans that could potentially induce osteo/odontogenic differentiation of DPCs and experimentally validate the effects *in vitro*. A subsidiary aim was to investigate the anti-inflammatory effect of identified compounds against cytokines associated with pulpitis (Rechenberg *et al*. 2016) using an *ex vivo* pulpitis model (Yonehiro *et al*. 2012, 2013).

Methods

Dataset selection

A search of the GEO database was performed using the keywords: 'dental pulp stem cells' 'DPCs,' 'differentiation,' 'lineage' 'osteogenesis', 'odontogenesis' 'osteogenic' and 'odontogenic.' Datasets were assessed for inclusion in the study using the criteria: cell type, induction of osteo/odontogenic differentiation, and the presence of both an undifferentiated control and differentiated treatment group.

Connectivity mapping

The Series Matrix Files (SMF) for the selected GEO datasets contained gene expression levels for all the probes used in the microarrays. These were downloaded and opened in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Paired t-tests were performed on the gene expression levels measured in undifferentiated controls and cells that had undergone osteogenic/odontogenic differentiation. Genes with significant ($p<0.001$) differential expression were selected and their fold change was calculated (Malcomson *et al.* 2016). Genes were given a score of +1 or -1, depending on whether they were upregulated or downregulated.

The datasets used in this study were identified from GEO platform 6244 (GPL6244). To be compatible with ssCMap, the significantly differentially-expressed genes were converted to GPL96 probe IDs (See details in supplementary material). Connectivity mapping was carried out with the signature genes outlined in Table 1 using Zhang and Gant's ssCMap (Zhang & Gant 2009) with default settings, which included a false connection tolerance of 1.0. Compounds with a significant positive connection score were considered for further analysis. The biological functions

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of the differentially expressed genes outlined in Table 1 were analysed using Ingenuity Pathway Analysis (IPA) software (Qiagen, Hilden, Germany).

Isolation and culture of DPCs

DPCs were derived by explant culture from immature permanent third molar teeth obtained in accordance with French ethics legislation. Cells were grown in minimal essential medium with L-glutamine supplemented with 10% foetal bovine serum (FBS), 100UI/mL penicillin and 100µg/mL streptomycin and maintained in an incubator at 37C°and 5% CO_2 throughout. DPCs were seeded in 6-well plates at a density of $3x10^4$/mL and treatment protocols commenced when cells were 70-80% confluent. Treatment protocols included, treatment with either α-MEM alone, osteogenic media (OM) containing 2mM β-glycerophosphate (BGP) or α-MEM supplemented with the predicted compound.

MTT assay

DPCs were seeded in 96-well plates at a density of $3x10^4$/mL and treated with normal media or predicted compound at concentrations ranging from 0.005mM to 10mM for 3, 5 or 7 days. After the relevant time period 10µL MTT solution was added to each well for 2h at 37°C. Media was aspirated and wells air-dried for 10 minutes. Then 200µL DMSO was added and plates were incubated at 37°C for a further 10 minutes. Samples were mixed and absorbance was measured at 510nm using a Tecan GENios microplate reader.

Alkaline phosphatase (ALP) activity assay

ALP activity of control and treated cells was assessed at 14 days. Cell lysis was performed with 25 µL radioimmunoprecipitation assay (RIPA) buffer on ice. An alkaline phosphatase diethanolamine detection Kit (Sigma Aldrich, St. Louis, Missouri, USA) was employed to perform the assay, as previously described (Winning *et al*. 2017). Briefly, the assay was a 96-well plate format, with 5µL of each cell lysate added to 240µL of reaction buffer. Following incubation for 20 minutes, the substrate p-nitrophenylphosphate (pNPP) was added (5µL of 0.67M pNPP solution). A negative control of 245µL reaction buffer with 5µL of 0.67M pNPP was also included. Absorbance was measured over 20 consecutive reads at 405nm.The ALP results were normalised to protein concentration determined using Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Alizarin red staining

The mineralisation of treated and untreated cells was assessed with the Alizarin Red (AR) staining assay. Cells were fixed in ice cold 70% ethanol for 30 minutes at room temperature. Staining was performed with 1mL 2% AR solution (pH adjusted to 4.1-4.3 using NaOH) for 45 minutes at room temperature in the dark. Cells were destained with 1mL 10% cetylpyridinium

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chloride in 10mM disodium hydrogen phosphate (pH 7) for 60 minutes. Absorbance was measured at 570nm.

Real-Time qPCR

RNA was extracted from treated and untreated cells using Qiagen RNeasy RNA Extraction Kit as per the manufacturer's instructions. cDNA synthesis was carried out with SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) using 500 ng RNA. Real-time qPCR was carried out to quantify osteo/odontogenic genes ALPL, RUNX2, COL1A1, DSPP, DMP-1 and SPP1. A panel of genes identified by differential expression analysis namely: PELI 1, ERBB3, NARS and RABGAP1 were also analysed. GUSB and B2M were used as reference/housekeeping genes (Supp Table 1). qPCR was performed using a Stratgene Mx3005P (Agilent Technologies, Santa Clara, CA, USA) and data was recorded by MxPro software (Agilent Technologies). The thermal profile consisted of an initial period of 2 minutes at 50°C and 10 minutes at 95°C, followed by 42 cycles of 30 s at 95°C and 1 min at 60°C. For each primer a control with no template cDNA and a control with no reverse transcriptase were used.

Assessment of anti-inflammatory effect of the compound in an ex vivo pulpitis model

To assess the anti-inflammatory effect of the predicted compound, an *ex vivo* pulpitis model was established as previously described (Yonehiro *et al.* 2012, 2013). Briefly, DPCs and differentiated THP-1 cells were co-cultured in a 12-well transwell system (Corning Costar, Corning, NY, USA). THP-1 cells were seeded in the transwell upper chamber at the concentration of $1x10^5$ cells per well. THP-1s were differentiated into macrophages by 24 h, in the presence of 150 nM phorbol 12-myristate 13-acetate (PMA, Sigma) followed by 24 h incubation in RPMI medium. DPCs were seeded in the lower chamber at a concentration of $2 x10^5$ cells per well. Cells were prepared in the transwells independently and just before the start of the experiments, both upper and lower chambers were combined as a co-culture, being separated only by a porous membrane (0.4 mm pore diameter). To validate the co-culture system and to confirm previous findings that cytokines levels differ when cells are in monolayer compared to co-culture (Yonehiro *et al.* 2012), a set of experiments were performed in which differentiated THP-1 and DPCs in monolayer and co-culture were treated with 1µg/mL LPS for 24 h and the levels of the cytokines IL-8 and IL-B were measured in the cell supernatant using Duoset ELISA kits (R&D Systems, Minneapolis ,MN, USA).

The co cultured THP-1 macrophages and DPCs were then treated with 1µg/mL ultrapure *E. coli* LPS with or without the compound/drug of interest for 24 h. Cytokines were measured from cell supernatants collected from both chambers of the co-culture system using a magnetic Luminex

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assay kit (R&D Systems) to simultaneously measure; IL-6, MMP-9, IL-8, CCL21, IL-1β, IFN gamma, angiogenin, and angiopoietin.

To test the effect of the selected compound on the viability of differentiated THP-1 macrophages an MTT assay was performed as described above for DPCs, except that THP-1 cells were seeded at a density of $3X10^4$ and treated with the predicted compound at a concentration of 0.05mM for 24h.

Statistical analysis

Data were summarised as means ± standard deviations (SD) and analysed using GraphPad Prism software, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Means were compared using the *t- test or ANOVA* followed by a Bonferroni post-test with the level of significance set at $p \leq 0.05$.

Results

Connectivity mapping

The GEO database search identified an appropriate dataset (GSE44677), which was available on the GPL6244 platform. Differential expression analysis and conversion of probe IDs to the GPL96 platform (as explained in supplementary material) resulted in the identification of 11 genes (Table 1) with a role in osteo/odontogenic differentiation. These were used as the gene expression signature to interrogate the connectivity mapping database. The ssCMap identified 0.1mM acetylsalicylic acid/aspirin (ASA) as the only drug with a significant positive connection score (Figure 1). The connection score for ASA was 3.583, the set score was 0.262 and the p value was 0.0002. ASA, which is non-steroidal anti-inflammatory drug was therefore, selected for laboratory analysis to determine if its predicted role in osteogenic/odontogenic induction could be confirmed *in vitro*. To understand the biological function of the differentially expressed genes shown in Table 1, a function and pathway analysis was performed using Ingenuity Pathway Analysis (IPA) software. Analysis revealed the most significantly perturbed biological functions in the dataset to be cellular processes associated with cell morphology, development, movement, growth and proliferation among others (Figure 2).

Effect of ASA on cell viability

The viability of DPCs was assessed following treatment with ASA (0.005mM to 10 mM) using the MTT assay. At low concentrations (< 1mM), which were suggested by the ssCMap, ASA did not affect the viability of DPCs or THP-1 cells, however, it was significantly affected at higher ASA concentrations (Figure 3. A, B, C & Supp Figure 1).

ALP Activity and mineralisation assays

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The treatment of DPCs with either 0.05mM ASA or osteogenic media (OM) resulted in significantly higher ($p<0.001$) ALP activity than untreated cells (Figure 3 D). Similarly, Alizarin red staining demonstrated higher mineralisation in ASA and OM treated cells compared to cells treated with αMEM for 14 days, $p<0.001$ (Figure 3E&F). There was no difference in the osteogenic effect between 0.05mM or 0.5mM ASA, and the lower concentration was used for the rest of the experiments as this approximately equates to the serum level of ASA after low dose 75 mg aspirin treatment (Patrignani *et al*. 2014).

qPCR and gene expression analysis

Treatment with 0.05 mM ASA induced higher expression of the DMP1 gene compared to control culture after 24 h. At day 7 only the DMP1 and DSPP genes were increased compared to control, however, expression of the osteogenic markers RUNX2, COL1A1, SPP1 and ALPL did not change significantly (Figure 3 G&H).

To verify the expression of the genes identified by differential expression analysis, four were selected for qPCR quantification. As shown in Supp Table 2, all the genes were expressed in DPCs and ASA treatment modulated their expression in a way that was relatively comparable to their fold change shown in Table 1.

Regulation of inflammatory cytokines with ASA

To assess if ASA at a concentration of 0.05mM exerted an anti-inflammatory effect, the level of a panel of cytokines previously reported as biomarkers for pulpitis (Rechenberg *et al.* 2016) was measured in an *ex vivo* pulpitis model using multiplex array. The production of cytokines in the co-culture pulpitis model was significantly higher than in THP-1 macrophages or DPCs grown as monolayers (Supp Figure 2). ASA treatment resulted in significant downregulation of the pro-inflammatory cytokines IL-6, MMP-9 and CCL21, but had no significant effect on expression of angiogenin, angiopoietin, IL-1 β, IFN gamma and IL-8 in the *ex vivo* pulpitis model (Figure 4).

Discussion

The main finding of this study was that connectivity mapping identified aspirin as a compound that could induce odontogenic differentiation. The gene expression signature of aspirin was shown by ssCMap to have the strongest correlation of any drug or compound in the reference database to the gene expression changes identified as occurring during osteo/odontogenic differentiation of DPCs. Aspirin is a prime candidate for drug repurposing due to its well-documented safety profile in humans. As such, the possible use of aspirin to repair damaged dentine in cases of deep caries and associated pulp inflammation could be particularly important. Connectivity mapping is a powerful approach that has principally been used to identify new applications for existing

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therapeutics, termed drug repurposing. The majority of previous CMap studies have focused on identifying whether an established drug, classically used to treat a certain disease or condition, shows promise in the treatment of an entirely unrelated disease or condition. For instance previously unrecognized analgesic and antinociceptive properties had been identified for the anti-hypertensive drug phenoxybenzamine (Chang *et al*. 2010). The results of the current study demonstrate that connectivity mapping can be used to identify compounds that can induce the differentiation of DPCs. In a similar recent study, parbendazole, primarily used as an anthelmintic agent, was identified as an inducer of osteogenic differentiation in bone marrow derived mesenchymal stem cells (Brum *et al*. 2015).

Support for the ability of aspirin to induce differentiation of stem cells comes from recent studies demonstrating osteogenic differentiation of DPCs (Yuan *et al*. 2018) and PDL stem cells (Abd Rahman *et al.* 2016). It was demonstrated *in vitro* and *in vivo* that aspirin, even at low concentrations, improved osteogenic differentiation of SHED via the (TERT)/Wnt/β-catenin pathway (Liu *et al*. 2015). In these studies the use of aspirin was not underpinned by a rationale that supported its choice because it was superior to any other drug but rather they were exploratory studies of the effects of aspirin. In the current study the connectivity mapping approach identified aspirin as the highest scoring drug or compound matching the gene signature for osteo/odontgenic differentiation.

To validate the connectivity map findings, *in vitro* studies were performed to confirm the osteo/odontogenic effect of ASA. Upregulation of the odontogenic genes DMP-1 and DSPP occurred as well as evidence of mineralisation in response to a low concentration of 0.05 mM ASA. The results, however, showed no changes in the expression of the known osteogenic genes RUNX2, SPP1 and COLA1. Similar findings have been reported in a study investigating the osteo/odontogenic differentiation of DPCs (Loison-Robert *et al*. 2018). DMP-1 and DSPP play important roles in odontoblast differentiation and dentine formation (D'Souza *et al*. 1997); however, their presence in bone and other calcified tissues makes it difficult to use them solely as odontoblast markers. The identification of the odontoblast-like cell phenotype remains challenging due to a lack of unique molecular or morphological markers (Duncan *et al.* 2019) and most studies use a panel of osteo/odontogenic genes as markers. Our results are in line with previous studies that demonstrated upregulation of DSPP and DMP-1 during odontoblast differentiation (He *et al.* 2008). An increase in SPP1 also occurred, but this did not reach statistical significance, however, since SPP1 is a late marker of osteo/odotogenic differentiation, its level may have increased beyond day 7 in the present study. RUNX2 is a master regulator of osteoblast differentiation and although its exact role in odontogenesis is not known studies have shown that downregulation of RUNX2 is a prerequisite for cells to proceed towards the odontoblast lineage

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(Gaikwad *et al*. 2001, Li *et al*. 2011, Widbiller *et al*. 2016). Therefore, the low levels of RUNX2 gene expression observed at 7 days may indicate odontogenic, rather than osteogenic differentiation of DPCs. Similarly, COL1A1 gene expression was previously reported to decrease with time during odontogenic differentiation of DPCs treated with Biodentine (Septodont, Saint-Maur-des-Fossés, France) (Zanini *et al*. 2012, Widbiller *et al*. 2016) and the results at 24 hours and 7 days are in line with this trend.

The mechanism by which ASA induces osteo/odontogenic differentiation of DPCs is not known, but it is likely to be downstream of the genes that have been identified by our differential expression analysis. The IPA analysis showed these genes to have cellular functions related to development, growth and proliferation, and could therefore contribute to odontogenic differentiation of DPCs. The genes, RABGAP1 and PEL-I, are associated with autophagy (Popovic *et al*. 2012) and the NF-kB signalling pathway (Chang *et al.* 2009). These pathways have been shown to regulate odontogenic differentiation of DPCs. Autophagy has previously been shown to positively regulate odontogenic differentiation of DPCs (Pei *et al*. 2016,Yang *et al*. 2015), whereas NF-kB signalling has been reported to contribute either positively or negatively to this process (Feng *et al*. 2013, Hozhabri *et al*. 2015). NARS, which was also among the upregulated genes, is an important mediator of the effect of fibroblast growth factor on bone formation, by enhancing osteoblast survival and proliferation (Park *et al*. 2009). Among the genes identified as downregulated by the differential expression analysis was ERBB3, a receptor for neuregulin 1. This gene was previously shown to be downregulated during osteogenic differentiation of mesenchymal stem cells (MSCs) by Wnt3a, indicating cross talk between ERBB3 and Wnt3a in MSCs osteoblastic differentiation via Wnt/B Catenin signalling (Jullien *et al*. 2012). Thus, functions of some of the genes identified in our gene signature are relevant to osteo/odontogenic differentiation but further studies should explore their specific roles in DPCs.

A connectivity mapping approach to identify drugs that could be repurposed for the differentiation of stem cells appears promising, however, the process currently has limitations. There is a paucity of publicly available microarray data for differentiating cells, especially a niche cell type such as DPCs. Another disadvantage is that microarray probe IDs have to be converted to GPL96 to be compatible with ssCMap. This can result in the loss of probes and could affect the range of compounds identified by ssCMap. Nevertheless, the connectivity mapping discovery of aspirin although unexpected, is highly relevant to a proposed potential clinical application in pulp capping. Aspirin is a drug commonly used for the treatment of pain and inflammation and as an antiplatelet agent (Fuster & Sweeny 2011). The mechanisms by which it produces these effects are varied, but are in part via inhibition of prostaglandin synthesis (Vane 1971) and of NF-κB signalling (Kopp & Ghosh 1994). The anti-inflammatory effects of aspirin in these studies were

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observed at high therapeutic doses. The current study demonstrated that aspirin at a low concentration of 0.05mM, which induced odontogenic differentiation, reduced the levels of inflammatory cytokines IL-6 and MMP-9 that are well documented biomarkers for pulpitis (Rechenberg *et al.* 2016, Zanini *et al*. 2017). The results also demonstrated that aspirin treatment has no effect on the angiogenic growth factors angiogenin and angiopoietin and the cytokines IL-8 and IL-1β. These findings demonstrate a selective anti-inflammatory effect of aspirin at low concentration that merit further investigation.

Conclusion

Connectivity mapping, a web based informatics method, was successfully used to identify aspirin as a candidate drug that could modulate the differentiation of DPCs. Aspirin was shown to induce odontogenic differentiation in DPCs *in vitro* and this together with its anti-inflammatory effects, make it a potential candidate for vital pulp therapies. Future work to investigate appropriate delivery methods for *in vivo* application is warranted.

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Conflict of Interest statement

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Figure Legends

Figure 1 The sscMap output for the signature genes outlined in Table 1. The map is a plot of the distribution of candidate compounds that may enhance (right side) or suppress (left side) the desired effect (osteo/odontogenesis). Significant candidates are above the green line with either enhancing (right hand side) or reducing (left hand side) effects. The green line is a pre-determined threshold p-value calculated using 1/*N* where *N* is the number of reference datasets. In this case *N* is 3738 resulting in a p value of 0.0003, at which the green line is set. ASA corresponds to the dot above the green line (significant) on the right side (enhancer). The connection score for ASA was 3.583, the set score was 0.262 and the p value was 0.0002.

Figure 2 Graph showing molecular and cellular function analysis of the differentially expressed genes using Ingenuity Pathway Analysis (IPA). The minimum significance level scored as –log (p-value) was calculated with Fisher's exact test. The significance value associated with a function in global analysis is a measure for how likely it is that genes participate in that function.

Figure 3 Effect of ASA on DPCs viability and osteo/odontogenic differentiation: (A-C) MTT graphs showing that ASA has no effects on DPC viability at concentrations < 1mM during culture for 3 (A), 5 (B) and 7 (C) days. (D) DPCs treated with 0.05mM ASA or osteogenic media (OM) for 14 days showed high ALP activity compared to non-treated cells. (E&F) DPCs showed evidence of mineralization in response to ASA and OM, which, was significantly higher than untreated cells. Expression of osteo/odontogenic genes after exposure to 0.05 mM ASA quantified by qPCR showed: (G) DMP1 gene expression increased after 24 hours; (H) Both DMP1 and DSPP gene expression increased after 7 days. Data represent mean+/- SD; *** p <0.001, ** p <0.01, * p<0.05, as determined by *ANOVA* with Bonferroni post hoc correction. Results represent an average of three independent experiments.

Figure 4 Effect of ASA treatment on cytokine release in *ex vivo* pulpitis model: pro-inflammatory cytokines IL-6, CCL21 and MMP-9 were significantly reduced in response to treatment with 0.05mM ASA, while expression of the angiogenic factors angiogenin and angiopoietin 1 and cytokines, INF gamma, IL-Iβ, IL-8 was unchanged. Data represent mean+/- SD; *** p< 0.001, * p <0.05 as determined by *ANOVA-test* with Bonferroni post hoc correction. Results represent an average of three independent experiments.

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Table 1 DPCs osteo/odontogenic gene signature List of genes (1) upregulated and (-1) downregulated identified by differential expression analysis. Only these 11 genes were ssCMAp compatible and therefore were used as the gene signature.

GPL96_ID	Gene Symbol	Gene Title	Fold change	Fold Value	P Value	Conversion
204028_s_at	RABGAP1	RAB GTPase activating protein 1	1.0507	0.071418426	0.00014075	+1
218319_at	PELI1	Pellino homolog 1 (Drosophila)	1.173	0.23031762	0.000262363	+1
218040_at	PRPF38B	PRP38 pre-mRNA processing factor 38 (yeast) domain containing B	1.066	0.092466009	0.000326306	+1
219808_at	SCLY	selenocysteine lyase	1.051	0.071873327	0.000422738	+1
206497_at	C7orf44	chromosome 7 open reading frame 44	1.058	0.0818744	0.000712191	+1
200027_at	NARS	asparaginyl-tRNA synthetase	1.044	0.062898243	0.000903409	+1
220359_s_at	ARPP21	cAMP-regulated phosphoprotein, 21kDa	0.965	-0.051191504	0.000109973	-1
207399_at	BFSP2	beaded filament structural protein 2, phakinin	0.862	-0.214114885	0.000396292	-1
208415_x_at	ING1	inhibitor of growth family, member 1	0.915	-0.127681727	0.000494042	-1
202454_s_at	ERBB3	v-erb-b2 erythroblastic leukaemia viral oncogene homolog 3 (avian)	0.9284	-0.1017104776	0.00059818	-1
204416_x_at	APOC1	apolipoprotein C-I	0.9425	-0.085298083	0.00089721	-1

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Figure 1

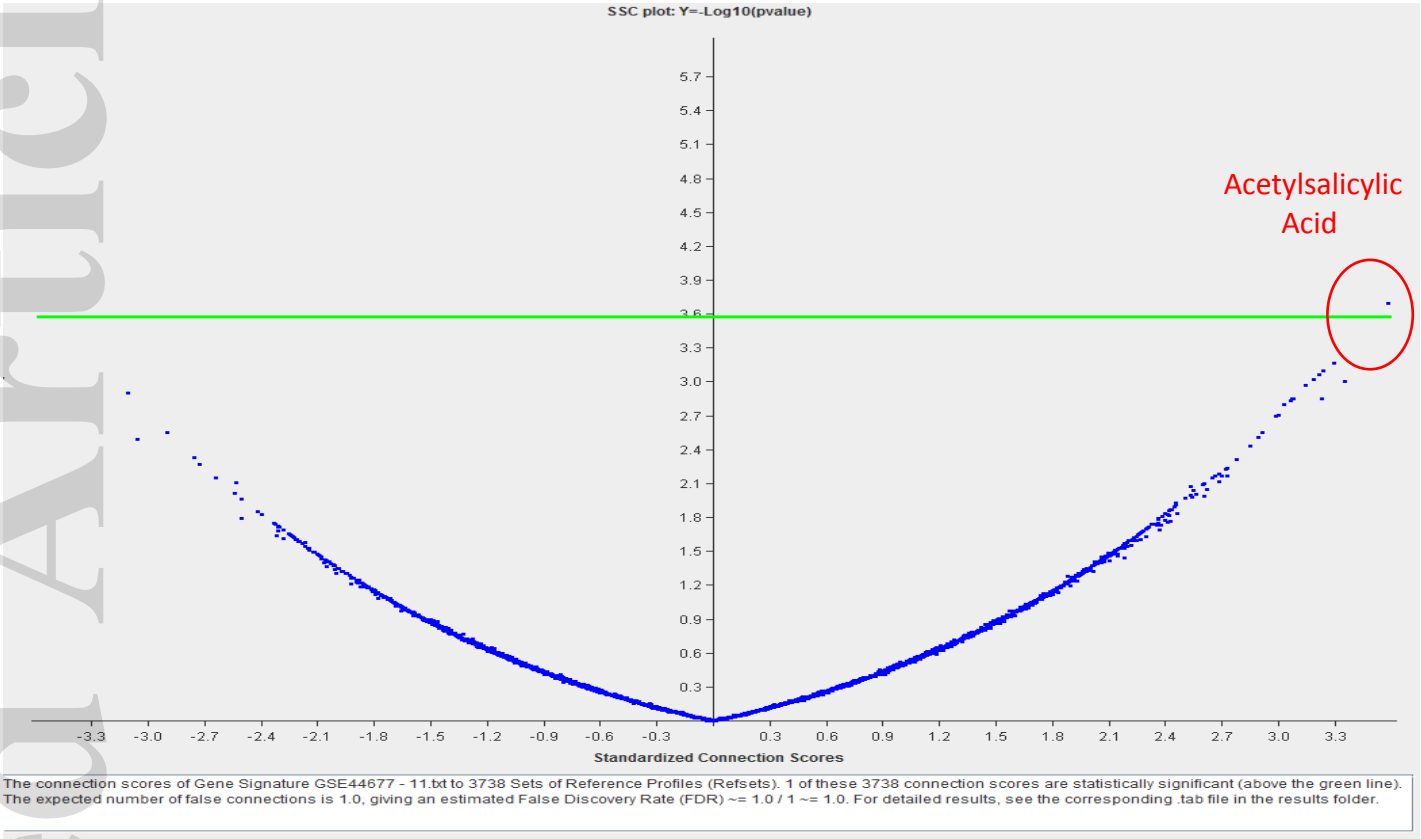

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Figure 2

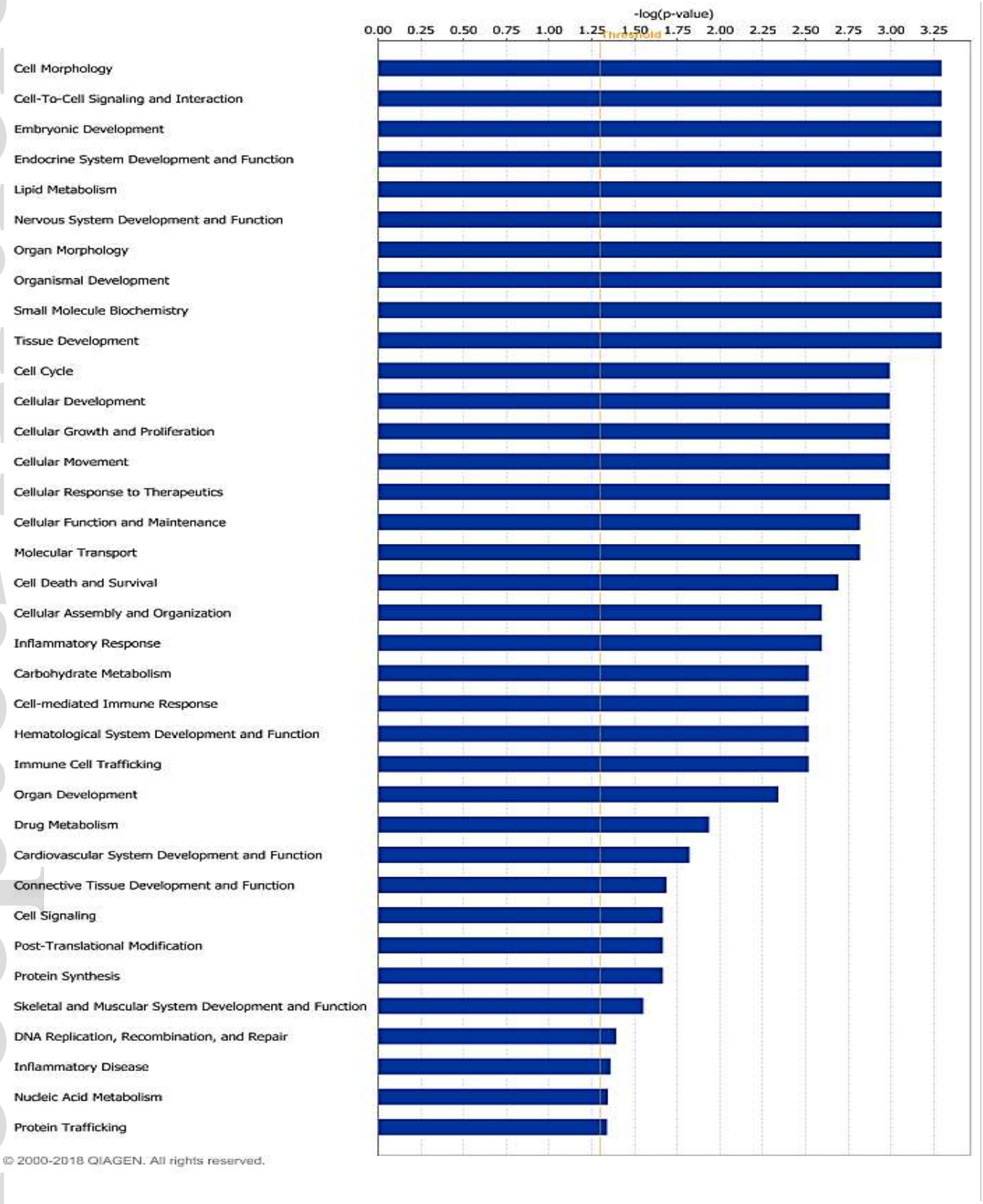

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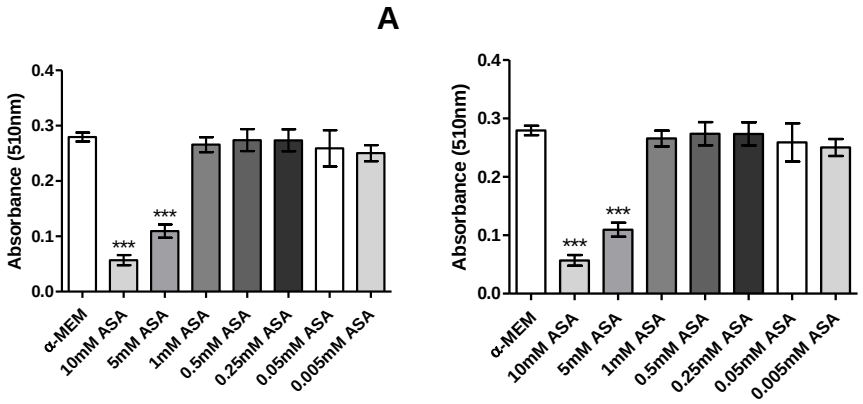
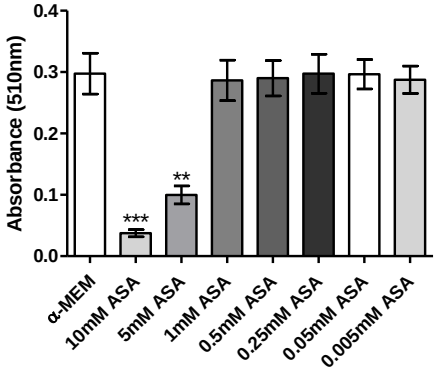
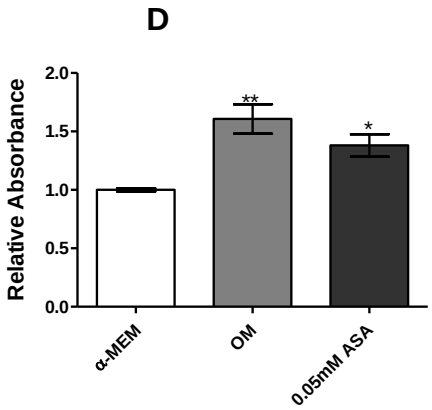
Figure 3

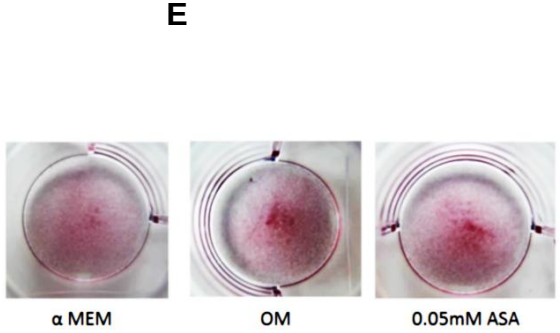

A
Absorbance (510nm)
0.4
0.3
0.2
0.1
0.0
***
***
α-MEM
10mM ASA
5mM ASA
1mM ASA
0.5mM ASA
0.25mM ASA
0.05mM ASA
0.005mM ASA

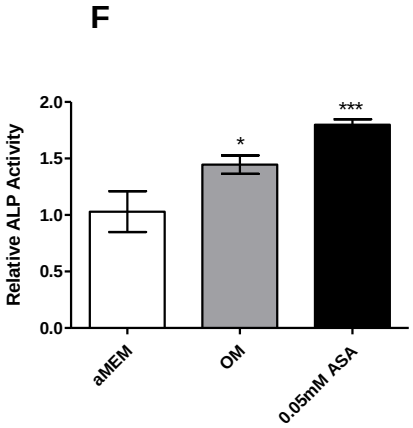
Absorbance (510nm)
0.4
0.3
0.2
0.1
0.0
***
***
α-MEM
10mM ASA
5mM ASA
1mM ASA
0.5mM ASA
0.25mM ASA
0.05mM ASA
0.005mM ASA

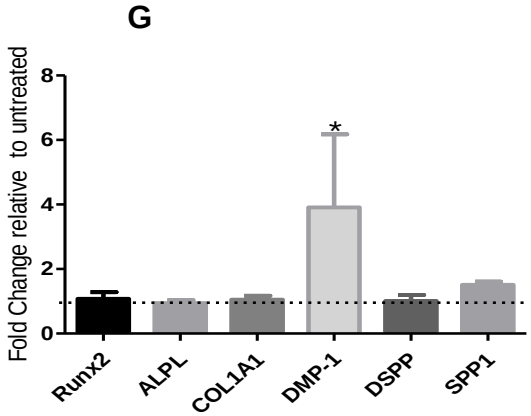
B


C
Absorbance (510nm)
0.4
0.3
0.2
0.1
0.0
***
**
α-MEM
10mM ASA
5mM ASA
1mM ASA
0.5mM ASA
0.25mM ASA
0.05mM ASA
0.005mM ASA


D
Relative Absorbance
2.0
1.5
1.0
0.5
0.0
**
*
α-MEM
OM
0.05mM ASA


E
α MEM
OM
0.05mM ASA


F
Relative ALP Activity
2.0
1.5
1.0
0.5
0.0
***
*
aMEM
OM
0.05mM ASA


G
Fold Change relative to untreated
8
6
4
2
0
*
Runx2
ALPL
COL1A1
DMP-1
DSPP
SPP1

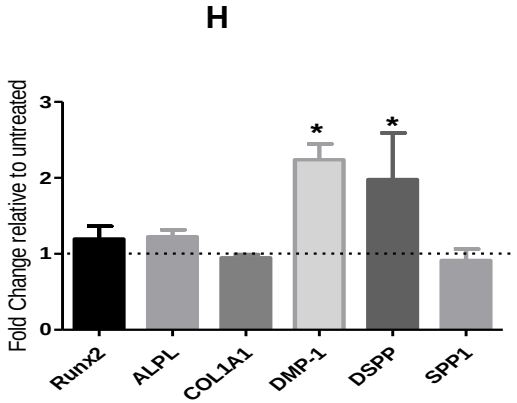

H
Fold Change relative to untreated
3
2
1
0
*
*
Runx2
ALPL
COL1A1
DMP-1
DSPP
SPP1

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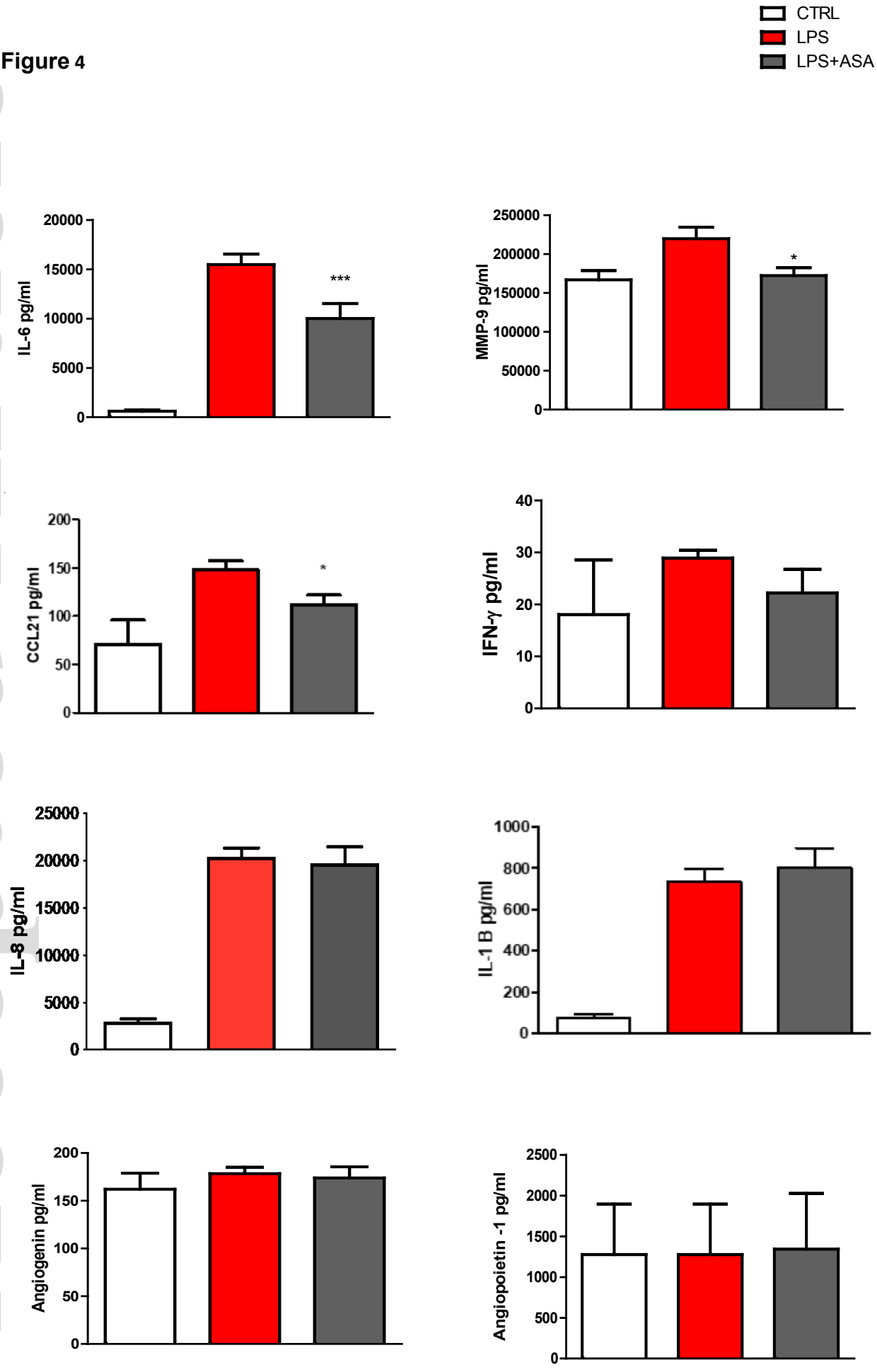
Figure 4



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