RESEARCH ARTICLE

IL-1 receptor antagonism and muscle gene expression in patients with type 2 diabetes

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ABSTRACT. *Background.* **We have previously reported that systemic blockade of IL-1β in patients with type 2 diabetes with anakinra (a recombinant human interleukin-1-receptor antagonist, IL-1Ra), lowered glycated hemoglobin improved beta-cell function and reduced circulating levels of IL-6 and CRP (7). To investigate the effects of IL-1Ra in insulin-sensitive tissue, gene expression levels in skeletal muscle from type 2 diabetic patients treated with IL-1Ra were analysed.** *Methods.* **Gene expression profiles in vastus lateralis muscle biopsies from five obese patients (BMI >27) were determined before and after 13 weeks of treatment with IL-1Ra (anakinra) using Affymetrix U133Plus2.0 GeneChips. Microarray data were normalized and analysed independently using four different algorithms; RMA, GCRMA, dChip and GCOS. Hypothesis tests were applied to the microarray data for each gene, and protein network analysis was used to identify biological networks/pathways affected by the treatment. Gene expression levels for candidate genes (COL1A1, CDKN1C, HSP70, HLA-A, IL-1 and IL-6) were determined by qRT-PCR in muscles of placebo- (n = 12) and anakinra-treated patients (n = 11).** *Results.* **The concordance of the variations of the transcripts identified as significantly regulated after IL-1Ra treatment was low. No significantly altered expression levels could be demonstrated after false discovery rate correction. The protein interaction network did not reveal any altered networks/pathways. None of the candidate genes, quantified by qRT-PCR, were significantly altered when comparing the number of transcripts before and after treatment for each individual.** *Conclusion.* **Treatment with IL-1Ra did not significantly affect gene expression levels in skeletal muscle in this limited and selected sample of obese patients with type 2 diabetes. Larger studies might confirm the lack of effect of anakinra on muscle tissue gene expression.**

Keywords: cytokine, insulin resistance, stress signalling

Type 2 diabetes occurs when beta-cell function and beta-cell mass fails to compensate for insulin resistance [1, 2]. This relative insulin deficiency leads to glucose intolerance and eventually hyperglycemia. Beta cell loss is mainly due to an apoptotic process, and several factors have been proposed to mediate this process such as chronic gluco-lipotoxicity, endoplasmic reticulum stress, oxidative stress, inflammation and cytokines [3, 4].
Sub-clinical, low-grade inflammation is associated with type 2 diabetes. Genetic and epidemiological studies, animal models and a recent clinical intervention trial indicate that an elevated expression of immune mediators in either pancreatic islets or insulin-sensitive tissues contributes to the pathogenesis of type 2 diabetes [5]. These biomarkers of inflammatory response include: raised circulating levels of acute phase proteins such as C-reactive protein (CRP) and cytokines/chemokines, *e.g.* interleukin (IL)-1, IL-6 and tumor necrosis factor α (TNF-α) [5, 6]. A placebo-controlled, double-blind, clinical trial showed that blockade of IL-1 with anakinra (a human recombinant IL-1-receptor antagonist, IL-1Ra) in patients with type 2 diabetes improved glycaemia and beta-cell secretory function [7]. Significant changes in systemic, low-level inflammation after anakinra treatment were also noted but were not correlated with the observed, primary, glycemic outcome. Although the above mentioned study reported no significant regulation of two transcripts involved in insulin signalling (Glut4 and PGC-1), we reasoned that changes in systemic, low-level inflammation might impact stress-signalling in

insulin-sensitive tissues and that these changes would be detectable by a larger microarray study.

A role of *in vivo* IL-1 antagonism in insulin signalling in peripheral tissue or the inflammatory state seen in type 2 diabetes has been described [8-10]. Furthermore, a few studies have investigated the effect of IL-1 on insulin sensitive tissue *in vitro* [11, 12]. However, the influence of attenuated IL-1 signalling in muscle tissue, responsible for 80% of insulin-stimulated glucose uptake, has so far not been investigated in detail in humans. We hypothesized that *in vivo* antagonism of IL-1 signalling in patients with type 2 diabetes, would alter expression levels of genes involved in stress signalling in skeletal muscle.

Gene expression levels were compared by oligonucleotide microarray analysis in skeletal muscle biopsies from five patients before and after IL-1Ra treatment. Furthermore, muscle gene expression levels of selected candidate genes were analysed using qRT-PCR in 11 patients in the anakinra-treated group and compared to those of 12 patients in the placebo group.

METHODS

Subjects

This placebo-controlled, double-blinded, parallel-group study recruited 70 patients with type 2 diabetes, of whom 67 patients completed the study *(figure 1)*. Patients received either once-daily recombinant human interleukin-1-receptor antagonist (100 mg of anakinra donated by Amgen, CA, USA) or placebo, by subcutaneous self-administration, in the morning, for 13 weeks. Patients con-

tinued their baseline anti-diabetic therapy, diet, and other lifestyle habits. Patients were recruited through two participating outpatient clinics, the Steno Diabetes Centre, Denmark, (31 patients) and the University Hospital Zurich, Switzerland (39 patients). Detailed information about the clinical study has been described earlier [7].

Inclusion criteria were, in brief, > 20 years of age, type 2 diabetes according to the American Diabetes Association criteria, with a diabetes duration of more than three months, $BMI > 27 \ kg/m^2$ and a glycated hemoglobin level > 7.5%. Exclusion criteria were: presence of auto-antibodies to glutamic acid decarboxylase 65 or islet cell antibody 512, current infections, fever, ongoing treatment with antibiotics or anti-inflammatory drugs, or a glycated haemoglobin level > 12%.

All patients were asked, on a voluntary basis, to participate in donation of a biopsy from the vastus lateralis muscle before and after the treatment. A total of 23 out of the 67 patients volunteered for these biopsies.

We expected patients that experienced a reduction in their systemic IL-6 levels after treatment to exhibit the most significant changes in gene expression levels in muscle tissue. Thus, the inclusion criterion for the microarray analysis was any reduction in systemic IL-6. The five (out of the 11 patients, see *figure 1*) selected for the microarray analysis responded to the treatment as indicated by significantly lowered systemic IL-6 levels (4.5 ± 0.35 ng/L in average at baseline to 2.7 ± 0.57 ng/L after treatment, p = 0.001).

RNA preparation

The muscle biopsies were obtained from the vastus lateralis after completion of an oral, beta-cell stimulation test.
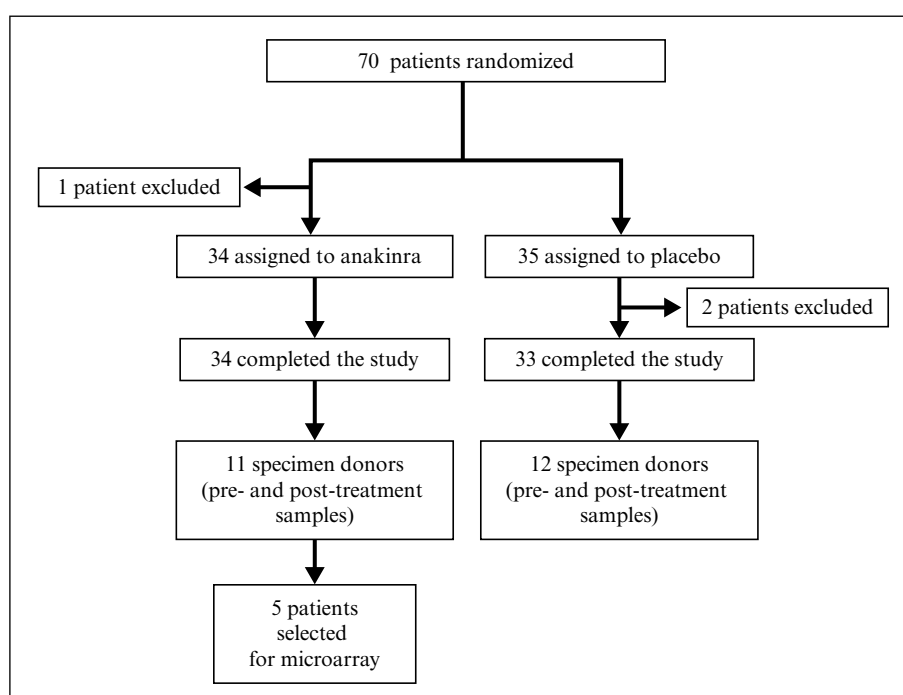

Figure 1
Trial design chart. Seventy patients were randomised to either placebo- or anakinra-treatment. Sixty-seven patients completed the 13 weeks of treatment. Out of these, 11 patients from the anakinra-treated group and 12 patients from the placebo group provided a muscle specimen at baseline and after 13 weeks of treatment.

They were immediately frozen in liquid nitrogen and stored at - 80°C until analysis. Total RNA was extracted using the Tri Reagent kit, following the manufacturer's instructions (Sigma-Aldrich Denmark, Brøndby, Denmark). cDNA was synthesized using RevertAid™ H minus First Strand cDNA Synthesis kit (Fermentas, Maryland, USA).

Oligonucleotide microarray analysis

RNA integrity was assessed by microfluidics-based analysis (Bioanalyzer, Agilent Technology, Santa Clara, USA). Upon confirmation of RNA integrity, a total of 500ng of RNA was labeled using the Single Round aRNA Amplification Kit (Ambion, TX, USA) and hybridized to Human Genome U133 Plus 2.0 arrays (Affymetrix, CA, USA). Hybridization, staining, and scanning were performed according to standard Affymetrix protocols. The hybridized arrays were scanned on an Affymetrix GeneChip® scanner 3000 7G.

Microarray data analysis

As an initial quality control, images of the hybridization intensities of each array were produced and the ratios of array/single outliers were calculated using dChip software. According to the software protocol, arrays with ≥ 5% outliers were discarded [13].

In recent years, it has become clear that the different algorithms available for microarray data analysis strongly affect the outcome of the analysis [14, 15]. Therefore, the raw data from the microarray experiment were pre-processed using the normalization algorithms: Robust Multichip average (RMA) and GCRMA (uses GC content of probes in normalization with RMA) algorithms from the Affy Bioconductor package version 1.7 (http://www.bioconductor.org) for the statistical software package R version 2.21 (http://www.r-project.org), Affymetrix GeneChip® Operating Software (GCOS) and dChip established by Li and Wong [13].

A paired, two-sided Student t-test was applied to all four data sets to compare altered gene expression levels before and after the treatment period in the five selected patients. Thus, patients served as their own controls. A ranking of the 50 most significantly regulated transcripts was generated.

Multiple hypothesis testing correction was applied to the gene expression lists provided by RMA and GCRMA. The "Multtest" function in the Bioconductor package version 1.7 for the statistical software package R version 2.21 (http://www.r-project.org) was used.

Protein network analysis

The 50 most significant IL-1Ra modified transcripts identified by RMA and GCRMA algorithms were analysed using the protein-protein network tool developed by Lage et al. [16]. This protein network tool consists of over 300,000, unique interactions between 8,500 human proteins constructed on data received from several sources including Kyoto Encyclopedia of Genes and Genomes (KEGG) protein-protein interactions, the Biomolecular Interaction Network Database (MINT) and the Biomolecular Interaction Network Database (BIND). Furthermore,

protein interaction information from 17 eukaryotic organisms were transferred and added to this human protein network [16]. For more information about this network program please refer to [16, 17].

The gene lists were converted to their respective protein names and entered into the network analysis tool. Interactions between proteins identified by the microarray analysis were taken to indicate that specific groups of genes or pathways were affected by the treatment.

Real-time PCR

Quantitative real-time PCR was carried out using TaqMan real-time PCR on cDNA from individual samples. For each primer and probe set, a standard curve was generated by a serial dilution of a cDNA synthesized from a pool of 10 cDNA samples received from the same subjects. Each sample was run in duplicate and the mean value of the duplicates was used to calculate the RNA expression level of each gene. The transcript quantity of each gene was normalized to peptidylprolyl isomerase A (PPIA). The following TaqMan gene expression assays (Applied Biosystems, CA, USA) were used: cyclin-dependent kinase inhibitor 1C (CDKN1C) (Hs00175938_m1), Collagen type 1A1 (Hs00164004_m1), IL-1 (Hs00174097_m1), IL-6 (Hs00174131_m1), major histocompatibility complex, class I, A (HLA-A) (Hs00740413_g1), heat shock protein 70 (HSP70) (Hs00359147_s1), PPIA (4333763T). The manufacturer's guidelines (Applied Biosystems) for the conditions for the PCR reactions were followed.

RESULTS

Clinical evaluation

In this subset of 23 patients, no significant differences were found in any of the baseline parameters measured (table 1) except for fasting plasma glucose levels. The anakinra-treated patients had slightly, but significantly, higher fasting glucose levels at entry than the placebo-treated group (p-value: 0.049). However, glycated haemoglobin showed no difference between the two groups. The five patients selected for the microarray study showed a trend towards lowered glycated hemoglobin levels after the treatment period ($9.0 \pm 0.36\%$ to $8.1 \pm 0.32\%$, p = 0.07).

Microarray analysis

Initially, the software dChip was used to determine the quality of each array after hybridization. All ten arrays were hybridized successfully. No array outlier levels were above 5%, indicating high quality arrays, with no contamination. Percentages of identified probe IDs (P-calls) between 30% and 60% are expected with the use of Affymetrix GeneChips. In the 10 arrays, P-calls between 41% and 56% were obtained, revealing that appropriate hybridization with high quality RNA had occurred.

Pre-processing of the raw data, normalized with either RMA, GCRMA, dChip, or GCOS resulted in four differ-

Table 1

Baseline clinical characteristics of the patients who donated muscle biopsies. Values are means ± SD. OAD: oral anti-diabetic drug.

	IL1-Ra (n = 11)	Placebo (n = 12)	P-value
Age (yr)	57 ± 8.2	60 ± 10.9	0.50
Sex (no.)			
- Male	9	11	
- Female	1	1	
Body mass index (kg/m^2)	31.0 ± 4.3	30.5 ± 2.3	0.32
Weight (kg)	98.5 ± 13.3	96.1 ± 5.9	0.57
Glycosylated hemoglobin (%)	8.9 ± 1.0	8.2 ± 0.8	0.11
Fasting plasma glucose (mM)	11.6 ± 2.6	9.2 ± 2.6	0.049
Fasting C-peptide (pM)	1 095 ± 255.7	909.9 ± 593.6	0.34
Diabetes duration (yr)	9 ± 6.7	10.5 ± 5.4	0.56
Cholesterol (mM)	4.8 ± 1.2	4.8 ± 1.2	0.95
Creatinine (μM)	87.5 ± 6.4	98.0 ± 19.0	0.095
Blood pressure			
- Systolic (mmHg)	147 ± 14.1	147 ± 20.7	0.96
- Diastolic (mmHg)	87.2 ± 6.7	83.2 ± 9.07	0.24
Use of antihypertensive medication (%)	64	75	
Antidiabetic treatment			
- OAD (%)	100	75	
- Insulin (alone or with OAD) (%)	18	50	

ent data sets (before and after treatment sample of five patients) each consisting of the gene expression indices of individual genes in all 10 arrays. Genes that were significantly regulated were identified by a paired t-test in each of the four data sets. Fold-changes were calculated as the average fold-induction between the baseline results and the results obtained after treatment.

To control for the rate of false positives among the differentially expressed genes identified by the paired t-tests, false-discovery rate (FDR) correction was applied to the data set [18]. No single gene could be classified as differentially expressed at a threshold of $p < 0.05$ after the FDR correction.

The results showed very different gene lists generated by each pre-processing method. Analysing the 50 genes with the lowest p-values revealed that not one single gene was represented in any of the four genes lists. Only a few genes showed a more than two-fold changes after anakinra treatment (data not shown).

Protein network findings

Although single genes with differential expression were not detected, groups of genes found in the context of protein interaction networks (modules) may be significantly enriched for the highest ranking genes according to the differential expression analysis before and after IL-1Ra treatment. Therefore, to detect putative interactions between expressed genes found by microarray analysis, the gene names were converted to their respective protein names and analysed in the protein-protein interaction network tool. Gene lists, including the 50 most significantly regulated genes from RNA and GCRMA, were analysed. None of the network analyses showed any direct connection between the proteins identified by the microarray analysis. Thus, no networks were identified among the proteins identified by the microarray analysis. This

strongly indicates that the identified genes are not part of a specific network of regulated genes. The gene list generated with dChip underwent the same protein interaction analysis and a few networks were found. Heat shock proteins (HSP70), platelet-derived growth factor receptor beta polypeptide, ribosomal genes and collagen proteins were represented in networks *(figure 3)*.

Realtime PCR

Six candidate genes (COL1A1, CDKN1C, HSP70, HLA-A, IL-1 and IL-6) were selected based on the results obtained from the protein network analysis (HLA-A, COL1A1), the microarray study (CDKN1C, COL1A1) or relevance to treatment (IL-1, IL-6).

Quantitative variations of five out of the six gene transcripts showed no significant differences between the anakinra group and the placebo group comparing before and after the treatment period. Only the gene transcript encoding for CDKN1C was significantly up-regulated in the anakinra group compared to the placebo group after treatment ($p = 0.001$). The gene transcrption levels were however only up-regulated by 20% compared to baseline *(figure 2)*. Four out of the six candidate genes (COL1A1, CDKN1C, HSP70, HLA-A) identified in the dChip gene lists (prior the FDR correction) were not differentially expressed by qRT-PCR. Taken together, the results indicate that treatment with anakinra had no effect on gene expression levels in skeletal muscle.

DISCUSSION

Blockade of IL-1 signalling with the IL-1 receptor antagonist anakinra in patients with type 2 diabetes has recently been shown to improve glycemia and β-cell secretory function, accompanied by a reduction in systemic inflammation
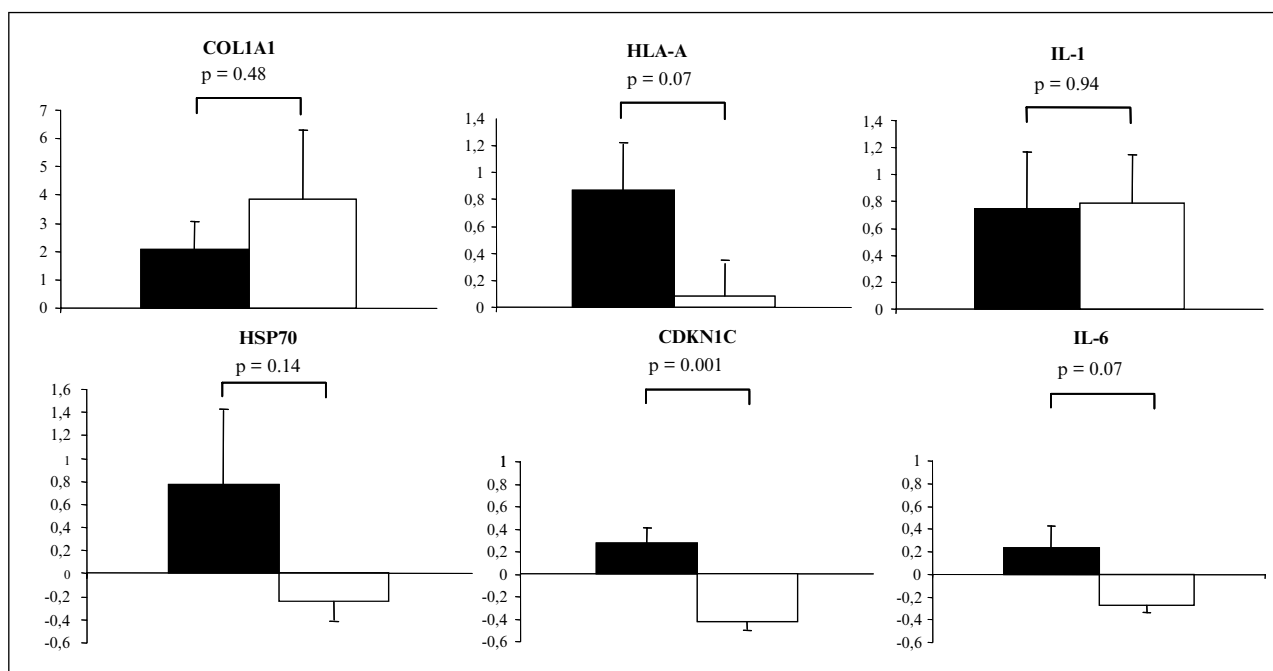
Figure 2
Gene expression in skeletal muscle from patients in the anakinra-treated group (n = 11) (black) and the placebo group (n = 12) (white). Collagen type 1A1, HLA-A, IL-1, HSP70, CDKN1C and IL-6 were quantified with qRT-PCR and normalized to PPIA. The results denote fold-changes between baseline and 13 weeks of treatment. A value of 1 represents a doubling of the gene expression level compared to baseline. Means ± SE.

markers [7]. In particular, systemic levels of IL-6 were lowered significantly in the patients treated with anakinra compared to placebo. We anticipated that the present microarray analysis of muscle tissue, including 47 000 different transcripts, would identify a range of genes affected by IL-1 antagonism. However, this analysis suggests that the documented changes in systemic, low-grade inflammation during anakinra treatment do not affect gene expression levels in skeletal muscle.

Gene expression levels were determined in vastus lateralis muscle from five patients with lowered systemic IL-6 levels under anakinra treatment. The microarray analysis indicated that no major gene expression changes occurred during the 13 week treatment period. Fold-changes were low (less than two-fold) and no significantly altered gene expression levels were found after multiple hypothesis correction. Furthermore, the qRT-PCR analysis of selected genes from samples including both the placebo group (n = 12) and the anakinra-treated group (n = 11) did not reveal any genes with altered expression levels.

It is possible that the lack of effect was due to underdosing of the drug and that higher doses could have evoked a greater response in skeletal muscle. The US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) regulatory authorities have approved subcutaneous administration of a maximum amount of 100 mg anakinra, once daily. Administration of a higher dose was therefore not possible. The clinical study did however clearly show that when stratifying the study group into tertiles according to body surface area, as a surrogate of drug distribution volume, those patients with the smallest body surface area obtained the greatest reduction in glycated haemoglobin levels [7]. This indi-

cated that higher dosages may lead to more pronounced clinical outcomes. None of the patients selected for the microarray analysis belonged to the tertile with the smallest body surface area.

The duration of the treatment in this study was 13 weeks, but significant changes in clinical parameters such as alterations in IL-6 and self-monitored plasma glucose levels were already being observed after four weeks [7]. Therefore, it is possible that the duration of treatment could have influenced the outcome of the gene expression analysis with compensatory mechanisms occurring to correct gene expression differences that might have been detected at earlier time points. However, no further biopsies were performed between week 0 and 13.

Increased systemic IL-1 levels, as observed in sepsis, have catabolic effects on muscle tissue [19], and studies have shown that IL-1Ra prevents sepsis-induced inhibition of muscle protein synthesis [20]. Although the circulating concentration of IL-1 is many folds higher in sepsis compared to the low-grade inflammation observed in patients with type 2 diabetes, these studies clearly illustrate that IL-1 is capable of affecting muscle tissue biology. In addition, both IL-1 and IL-1Ra have been identified by immunostaining in human myogenic cells [21].

IL-1 induces insulin resistance in adipocytes *in vitro* [11, 12]. Thus, it is possible that IL-1 induces insulin resistance in skeletal muscle by similar mechanisms. Furthermore, the IL-1Ra-mediated reduction of CRP and IL-6 might alter insulin resistance in skeletal muscle [22].

If the level of insulin resistance reflects a specific transcriptional pattern in skeletal muscle, changes in insulin resistance should be detectable by gene expression analysis. However, no significantly, regulated genes involved
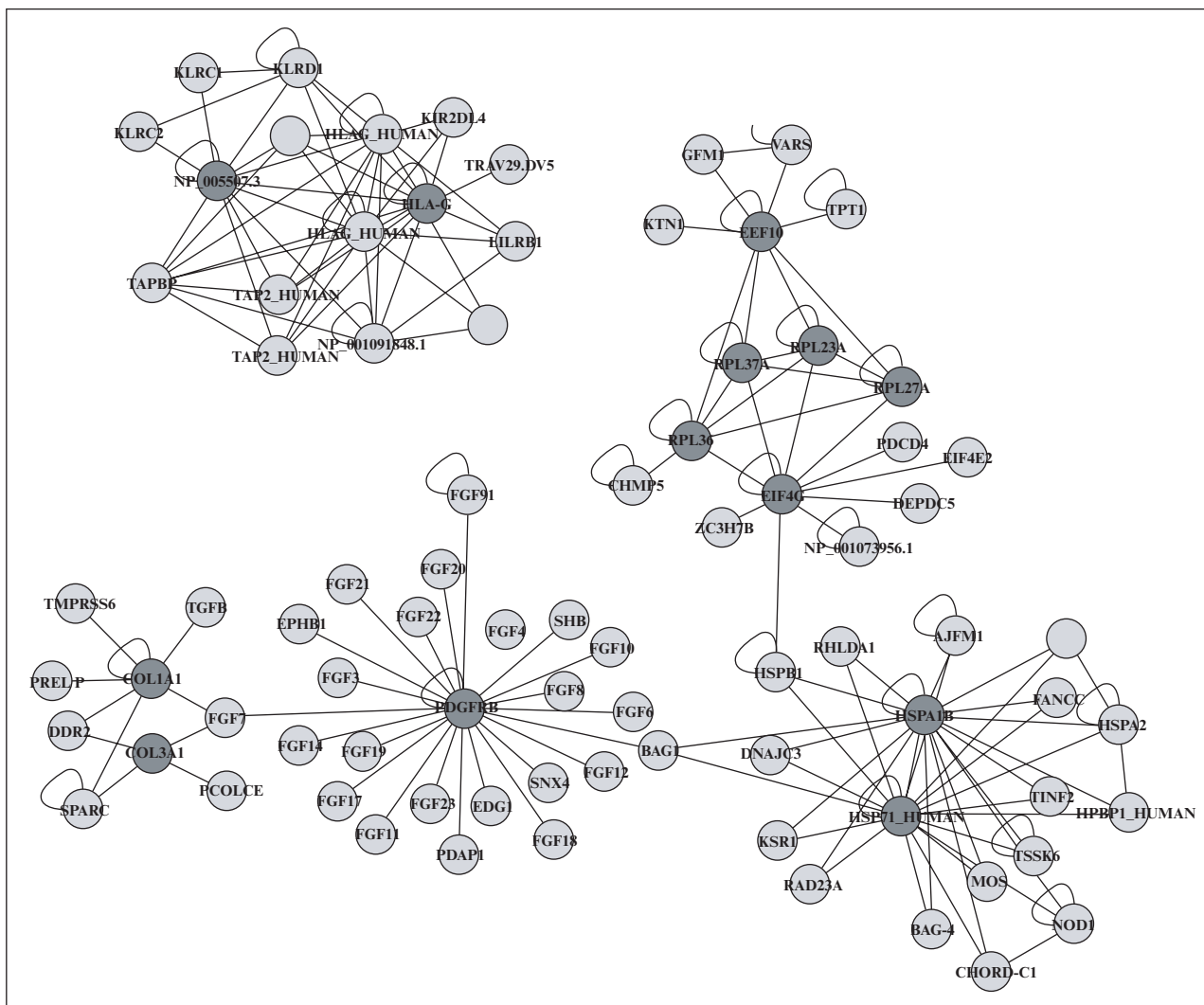
Figure 3

Protein networks putatively regulated by IL-1Ra. A network search algorithm was applied to the gene list produced by dChip (top 50 p-values by paired t-test). Black nodes represent proteins from the imported gene list and gray nodes represent interacting proteins identified by the network algorithm. Lines between the nodes denote a physical interaction between the two proteins involved.

in insulin signalling were found after anakinra treatment in this study. It is probable that low-grade inflammation may rather affect muscle enzyme activity *e.g.* by phosphorylation than gene transcription.

Several microarray studies have focused on muscle insulin signalling and type 2 diabetes. Sreekumar *et al.* investigated the effect of insulin treatment on skeletal muscle in patients with type 2 diabetes, and identified several genes that altered expression levels after insulin treatment [23]. It is however unclear whether these changes are caused by altered insulin signalling or simply due to correction of hyperglycemia [23]. Mootha *et al.* [24] as well as Patti *et al.* [25], using Affymetrix microarray technology, identified a set of genes involved in oxidative phosphorylation that were down-regulated in skeletal muscle biopsies obtained from patients with type 2 diabetes. However, another microarray study failed to identify gene expression changes or differences between normoglycemic insulin resistant *versus* insulin-sensitive groups [26]. This suggests that other mechanisms such as trans-

lational regulation or the phosphorylation of proteins involved in insulin signalling could play a more important role in insulin resistance than the expression level of specific genes *per se*.

In summary, this study is the first human microarray study that describes the effect of IL-1 blockade in skeletal muscle in patients with type 2 diabetes. The results show that intervention with anakinra in patients with type 2 diabetes does not affect gene expression levels in skeletal muscle despite significant reduction in markers of systemic inflammation. In addition, qRT-PCR validation in a greater number of placebo- and anakinra-treated subjects confirmed these findings. Hence, the possibility that the patients achieved improved glycemic control due to altered expression levels of genes involved in either the inflammatory response or insulin signalling in skeletal muscle is unlikely. Rather, these results support the findings of the clinical study [7] suggesting that the increased glycemic control in response to the anti-IL-1 treatment is mainly caused by improved beta-cell func-

tion and that insulin sensitivity in peripheral tissue is not affected at these doses of anakinra. Larger studies with higher doses of IL-1 receptor antagonist are needed to clarify this issue.

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