Detection of Growth Hormone Doping by Gene Expression Profiling of Peripheral Blood

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Context: GH abuse is a significant problem in many sports, and there is currently no robust test that allows detection of doping beyond a short window after administration.

Objective: Our objective was to evaluate gene expression profiling in peripheral blood leukocytes *in-vivo* as a test for GH doping in humans.

Design: Seven men and thirteen women were administered GH, 2 mg/d sc for 8 wk. Blood was collected at baseline and at 8 wk. RNA was extracted from the white cell fraction. Microarray analysis was undertaken using Agilent 44K G4112F arrays using a two-color design. Quantitative RT-PCR using TaqMan gene expression assays was performed for validation of selected differentially expressed genes.

Results: GH induced an approximately 2-fold increase in circulating IGF-I that was maintained throughout the 8 wk of the study. GH induced significant changes in gene expression with 353 in women and 41 in men detected with a false discovery rate of less than 5%. None of the differentially expressed genes were common between men and women. The maximal changes were a doubling for up-regulated or halving for down-regulated genes, similar in magnitude to the variation between individuals. Quantitative RT-PCR for seven target genes showed good concordance between microarray and quantitative PCR data in women but not in men.

Conclusion: Gene expression analysis of peripheral blood leukocytes is unlikely to be a viable approach for the detection of GH doping. (***J Clin Endocrinol Metab* 94: 4703–4709, 2009**)

GH is a potent anabolic hormone and plays an important role in body growth, development, and function throughout life (1). Its actions are exerted directly via the GH receptor or indirectly via IGF-I. GH is a prohibited substance under the World Anti-Doping Agency code of conduct (2, 3); however, it is widely abused among athletes. Detection of GH doping has been difficult because recombinant GH is indistinguishable analytically from endogenous GH, which itself undergoes wide fluctuations in the circulation (4, 5). Previous approaches to develop a GH doping test have examined changes in GH-responsive proteins in serum or in GH isoforms (6–8). The detection window for both approaches is short, being approximately 12–24 h for the isoform approach and 7–10 d for GH-responsive proteins (4).

GH regulates various components of the immune system (9, 10). *In vivo* studies have shown that GH administration increases natural killer cell activity (11). GH increases cytokine transcript levels in lymphoid cells *in vitro* (12). IGF-I generated in response to GH can act on immune cells giving rise to a secondary response (9). Therefore, peripheral blood represents a nonclassical GH target

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Abbreviations: ALR, Agilent log ratio; FDR, false discovery rate; Q-PCR, quantitative PCR; SDDA, stepwise diagonal discriminant analysis.

that may undergo characteristic changes in gene expression in response to treatment. We hypothesized that GH may induce effects on the peripheral blood transcriptome, which could be used as the basis for a GH doping test. The aim of this study is to determine whether a simple test for GH doping can be through the analysis of peripheral blood transcriptomes in humans.

Subjects and Methods

Study design

This study is drawn from a larger project analyzing the pharmacodynamics of markers of GH abuse in serum, the results of which have been recently published (6). Briefly, this was a double-blind, placebo-controlled study in male and female recreational athletes comprising an 8-wk treatment phase followed by 6 wk washout. Average ages of participants were 24.3 ± 2.4 yr (mean ± SE) for men and 31.7 ± 1.6 yr for women. This study comprised seven men and 13 women treated with GH 2 mg/d sc in whom peripheral blood mononuclear cells were obtained for gene expression analysis. GH (1 mg/ml Norditropin; Novo Nordisk, Bagsvaerd, Denmark) was self-administered at night. To minimize side effects, the dose was increased from 1 mg/d (first week) to 1.5 mg/d (second week) and then to the final dose of 2 mg/d for 6 wk. Participants attended the Clinical Research Facility at the end of each week, when the injection cartridge was changed and compliance checked by verbal reports and the volume remaining in the cartridge. Blood was collected at baseline (wk 0), during and after the final treatment point (wk 4 and 8), and after 6 wk washout (wk 14). RNA was prepared for microarray analysis at baseline and after the final GH treatment. The study was approved by the Human Research Ethics Committee, St. Vincent's Hospital.

Determination of serum IGF-I concentration

Serum samples were stored at −80 C before batch analysis. All samples for any individual were measured in the same assay run. IGF-I concentration (intraassay and interassay coefficients of variation of <4 and <9%, respectively) was measured by RIA after acid-ethanol extraction (13), using iodinated des(1-3)IGF-I (GroPep, Adelaide, South Australia) as radioligand.

RNA collection

RNA was extracted from white blood cells at the time of blood collection to avoid degradation within 6 h. Blood (14 ml) was collected by venipuncture, and after red cell lysis, RNA was extracted from the washed white cells using Trizol (Invitrogen, Waverly, Australia) with yields of 25–80 ng and stored under ethanol at −80 C before microarray analysis. Gel electrophoresis was performed to assess the integrity of each RNA sample. Before microarray analysis, RNA was purified by RNeasy mini spin columns (QIAGEN, Doncaster, Australia), and the concentration and quality was assessed by NanoDrop spectrophotometer and Agilent bioanalyzer, respectively.

Microarray and data analysis

Microarray analysis using the Agilent microarray platform was performed at The Ramaciotti Centre for Gene Function Analysis, University of New South Wales, Sydney. The Agilent Technologies (Santa Clara, CA) platform comprises a two-color design with two RNA samples labeled with either Cy3 or Cy5 and hybridized to the same 44K G4112F Agilent microarray. Each array contains about 41,000 unique noncontrol 60-mer probes. A total of 40 arrays on 10 slides were performed. Baseline (wk 0) and final GH treatment (wk 8) samples from seven men and 13 women were two-color labeled and hybridized to randomly chosen arrays. A dye swap was performed for each individual on a different slide to remove any bias from the labeling dyes.

To obtain the relative expression level of the two samples hybridized on each array, we used the Agilent log ratio (ALR), which is the log ratio of the processed red/green channel for each probe. ALRs were transformed to log base 2 and then scale normalized. After correcting for the dye swap, the following linear model was fitted to the data (14) from each probe independently: $y_i = \alpha_i + \beta_i + \varepsilon_i$, where y_i are the normalized expression ratios, α_i and β_i are the average ratios for the male and female samples, respectively, and ε_i is the residual error for probe *i*. To account for the additional correlation that is expected from dye-swapped technical replicates, we estimated the duplicate correlation among samples (15) and used the sample ID as a blocking variable in the linear model fit. To assess the extent of differential expression, an empirical Bayes, moderated *t* statistic (14) was performed. All analyses were performed using R version 2.7.1 (16) and the Limma package version 2.14 (14) from Bioconductor version 2.2 (17).

Microarray data are presented on a log2 scale where each unit represents a 2-fold change in expression level. The absolute fold change is used to denote changes in expression; *i.e.* a 2-fold increase is equivalent to a doubling of expression and a 2-fold decrease a halving of expression. For analysis of differentially regulated probes, we included only those with a log2 expression level of 6.0 or greater, signifying good expression above background. For comparison, the log2 expression for GAPDH was approximately 13.8.

For probe effect size comparison, we obtained the average expression level (*A* value) for each probe: $A = (R + G)/2$, where *R* and *G* are the background-corrected, log2-transformed mean intensities for the red and green channels, respectively. The average expression ratio (*M* value) for each probe was the ALR as before, where $M = R - G$. Thus, from *M* and *A* values, we obtained the normalized expression level for each channel *R* and *G* for each probe. Duplicate measurements were averaged to obtain expression level of each subject at baseline and wk 8. The variance of each probe attributable to GH was determined by averaging the variance of the two measurements for each individual. The variance of each probe between individuals was determined by averaging the expression level for each individual.

Data from individual channels were also used for multivariate analysis using stepwise diagonal discriminant analysis (SDDA) an algorithm developed by the Commonwealth Scientific and Industrial Research Organization. The method is an extension of diagonal discriminant analysis (18) that uses a forward stepwise approach to identify a discriminatory set of probes. It identifies linear combinations of probes that predict the samples from treatment time points from basal samples. The discriminatory power of individual probes or groups of probes identified was estimated by a cross-validation process. Under cross-validation, individual samples are removed and their status predicted using

SDDA applied to the remaining samples and the process repeated leaving out each sample (or individual) in turn.

Statistics

Where presented, *P* values are unadjusted for multiple testing by the positive false discovery rate (FDR) (19). Serum IGF-I levels and relative expression levels for microarray and quantitative PCR (Q-PCR) analysis are presented as the mean ± SE.

Q-PCR

RNA samples for seven men and 13 women were used for PCR analysis. cDNA was synthesized from 2 μg total RNA, 25 μg/ml oligo deoxy-thymidine (dT), 2.5 μM random primers (Promega, Sydney, Australia) using Superscript II (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Reactions were diluted 1:4 and stored at −80 C before Q-PCR. Validation of the selected genes was undertaken via TaqMan gene expression assays using the Applied Biosystems 7900HT (Applied Biosystems, Foster City, CA). For each gene, an assay was selected to amplify the region corresponding to the location of the relevant probe. Where a corresponding probe was not available, an alternative validated probe was selected for the gene. The housekeeping gene GAPDH was performed in parallel as control. Standard TaqMan cycling conditions were used, and all reactions were performed in triplicate. Changes in expression level by Q-PCR were calculated as $-\Delta\Delta CT$ (cycle threshold) and presented relative to average changes derived from ALRs as mean ± SE, on a log2 scale of relative expression (20). Increased expression has a positive value, no change zero, and decreased expression a negative value.

Genes were selected for Q-PCR validation from the top 200 probes. Targets were selected if more than one probe corresponding to a single gene was significantly regulated. Any further probes for that gene that was not in the original selection were also considered. Each individual probe was aligned against the genome to confirm selective targeting of an individual transcript.

Results

In both men and women, GH treatment induced a robust increase in serum IGF-I. The mean IGF-I concentration was elevated at wk 4 and remained elevated at wk 8, returning to baseline at wk 14 (Fig. 1). IGF-I levels in men and women increased 2.3- and 1.8-fold, respectively, at 8 wk.

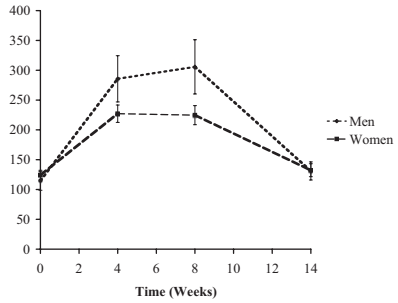

FIG. 1. GH-induced changes in IGF-I levels in microarray analysis subjects. IGF-I was measured at baseline (wk 0), during and after the final GH treatment (wk 4 and 8), and after 6 wk washout (wk 14). Results are shown as micrograms per liter IGF-I in serum in seven GH-treated men and 13 women, mean ± SE.

Gene expression changes

For both men and women, most probes cluster around zero in the log2 scale, indicating no significant change in expression (Fig. 2, A and D). The maximal ALR changes in both men and women for individual probes are approximately a doubling or halving. The distribution of *P* values was similar in men and women (Fig. 2, B and E) with 11.4 and 10.2% of all detected probes having $P < 0.05$, respectively. The relationship between *P* value and log fold change for all probes in men and women are summarized in Fig. 2, C and F. The *horizontal dashed line* indicates the *P* value threshold corresponding to a FDR of 5%, where there are 41 and 353 probes differentially regulated in men and women, respectively. The different numbers of arrays in men and women led to differences in the statistical power and, thus, the threshold for false discovery. There were seven and five differentially regulated probes with both an ALR more than ±0.585, corresponding to a ±1.5-fold change at a FDR less than 0.05, in men and women, respectively (*open circles* in Fig. 2, C and F). Thus, ALR for all probes revealed that the effect of GH was small.

Results from individual Cy3- and Cy5-labeled channels were used to estimate the inter-individual and GH effects across all probes. Analysis of either labeled channel yielded similar results. An estimation of the relative effects can be represented by plotting the within- and between-sample variances as a box plot (Fig. 3). For both men and women, the most extreme values are inter-individual effects, indicating that the largest effects are due to the difference in expression levels between individuals. In men, the median (0.103) and the range of the middle 50% of the data (0.059–0.192) for a GH effect was greater than the inter-individual effect, median 0.070 and range 0.038–0.134. However, the effect of GH remains similar to the variation between individuals. In women, the median was lower (0.077), and the range of the middle 50% of the variance (0.044–0.203) overlapped the range for men. Inter-individual variance in women was similar to men, median 0.078 and range 0.44–0.140. In brief, the effect of GH is similar to the magnitude of the variation between individuals.

Gender comparison

The maximal fold changes in expression induced by GH were similar in men and women. In men, this ranged from 2.09-fold increase to 1.96-fold decrease and in women from 2.02-fold increase to 1.82-fold decrease. In men, there were 36 probes with greater than 1.5-fold up-regulation and 13 with a similar degree of down-regula-

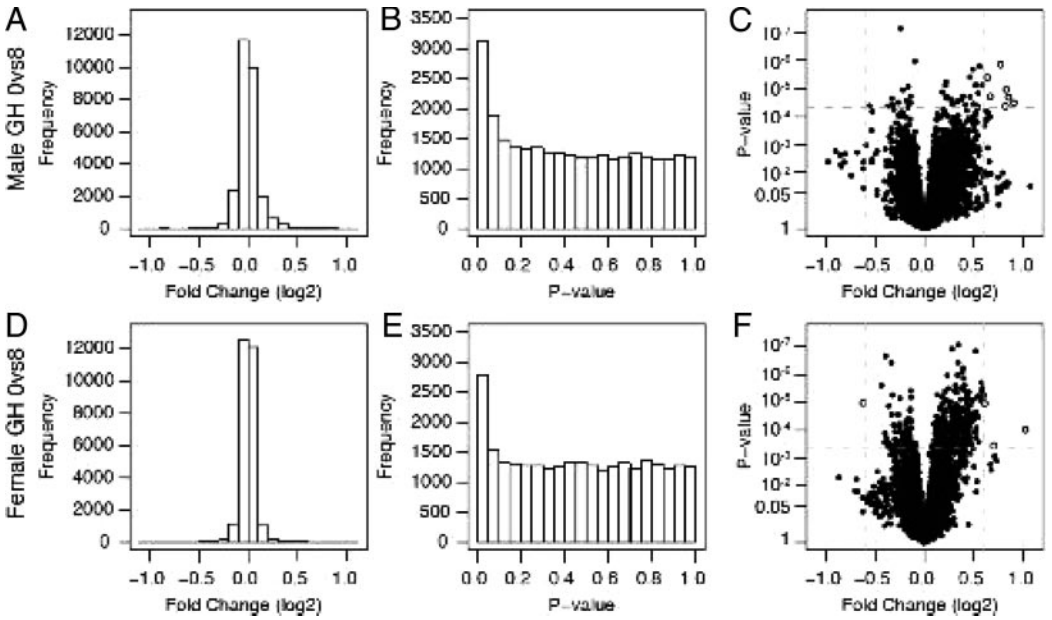

FIG. 2. Analysis by ALR in GH-treated men and women. ALR results for all probes on the G4112F microarray (see *Materials and Methods*). Range of log2 fold change *vs.* frequency, range of *P* value *vs.* frequency, and relationship between *P* value and log2 fold change for GH-treated men (A–C) and women (D–F) are shown. In C and F, the *horizontal lines* represent threshold $P < 0.05$ corresponding to a FDR of 5%, whereas the *vertical lines* indicate log2 fold change of 0.585 (equivalent to an absolute fold change of 1.5). The *open circles* represent the transcripts that show a greater than 1.5-fold change and FDR less than 5% in response to GH treatment.

tion, representing about 0.1% of all probes. For women, only 16 probes were regulated by more than 1.5-fold with eight up-regulated and eight down-regulated (Table 1). Approximately 11% of all detected probes were differentially regulated ($P < 0.05$ and log2 expression ≥ 6.0) in men and women (Table 1). In men, the majority, 1795, was up-regulated compared with 1336 that were down-regulated. In women, the results were similar; 2799 probes were differentially regulated, with 1465 up-regulated and 1334 down-regulated. More probes were identified in women at all cutoff values. At an FDR of 5%, 41 probes were identified in men, 28 (68%) up-regulated, and 353 in women of which 268 (76%) were up-regulated.

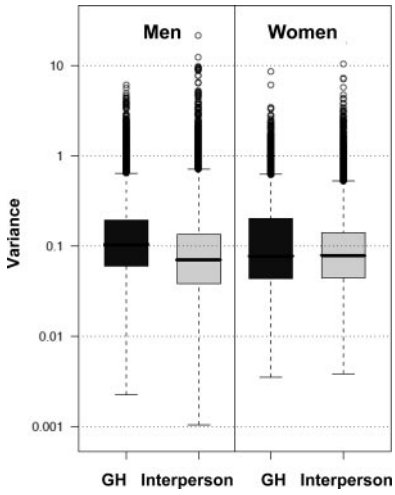

FIG. 3. Effect of GH compared to inter-individual variation on probes expressed above background. The effects of GH treatment relative to within-individual variation was determined as described in the *Materials and Methods*. Variation due to GH within individuals (GH) was compared with variation between individuals (Interperson). In the *box plots* for men and women, *solid lines* represent the median, the *box* represents the middle 50% of data, and *whiskers* represent 2 SD.

Probes common to men and women

We next determined whether there was overlap of up-regulated and down-regulated probes in men and women. At

TABLE 1. Probe regulation in GH-treated men and women.

Regulation	Treatment group	
	Women	Men
No. of probes		
Detected (% of all probes)	27412 (63)	27383 (63)
Up (%)[a]	13557 (49)	12997 (47)
Down (%)[a]	13855 (51)	14386 (53)
Maximum fold change		
Up	2.02	2.09
Down	1.82	1.96
Fold change >1.5		
Up	8 (50)	36 (73)
Down	8 (50)	13 (27)
Unadjusted $P < 0.05$		
Up (%)[a]	1465 (52)	1795 (57)
Down (%)[a]	1334 (48)	1336 (43)
FDR <0.05		
Up (%)[a]	268 (76)	28 (68)
Down (%)[a]	85 (24)	13 (32)

ALRs were used to identify differentially expressed probes as described in *Materials and Methods*. Results for all probes with an expression level of 6 on a log2 scale at either baseline or after treatment.

[a] Percentage of probes regulated up or down.

a FDR less than 0.05, no common probes were identified. Ranking the top 100 probes in men and women by fold change, three common up-regulated genes and three common down-regulated genes were identified. The three up-regulated genes were IGF-II, MED18, and PDK4. The three common down-regulated genes were AREG, ARG1, and CYYR1.

Discriminant probe sets

We applied a multivariate analysis approach using SDDA to identify groups of probes to discriminate between the baseline and GH-treated results for men and women. A group of three probes for men (data not presented) and one of five probes for women (data not presented) could discriminate between the two treatment points but did not have the largest average fold change. However, these groups of probes performed very poorly on cross-validation. This approach did not identify groups of probes that could discriminate between treatment in men or women.

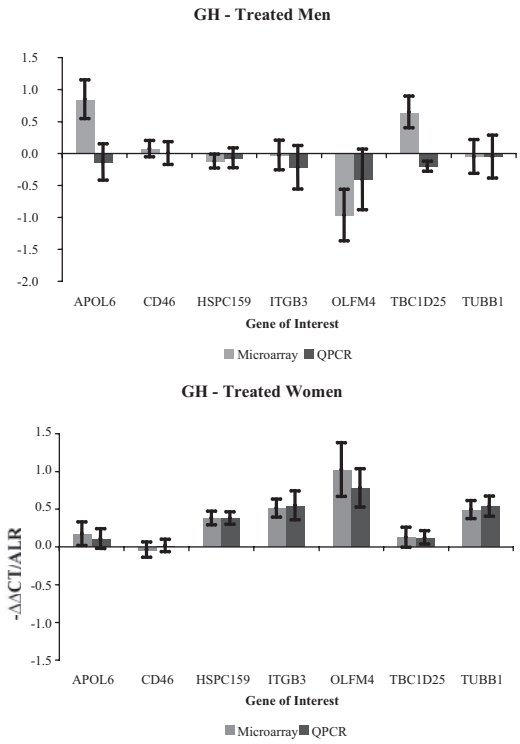

FIG. 4. Validation of target genes via TaqMan gene expression assay. For the seven targets identified, expression relative to the housekeeping gene GAPDH was calculated as described in the *Materials and Methods*. Q-PCR results are shown as $-\Delta\Delta$CT (cycle threshold) for men and women for the difference in expression between baseline and final treatment *vs.* ALR, mean ± SE. TaqMan gene expression assay IDs are as follows: GAPDH, Hs99999905_m1; CD46 Hs00387246_m1; OLFM4, Hs00197437_m1; APOL6, Hs00229051_m1; TBC1D25, Hs00412781_m1; HSPC159, Hs00204379_m1; ITGB3, Hs01001469_m1; and TUBB1, Hs00258236_m1.

Validation by Q-PCR

Six differentially expressed genes were selected on the basis of altered expression due to GH treatment for validation in men and/or women. An additional gene, CD46, was selected because it was unaltered by GH treatment in either men or women. In agreement with the microarray data, there were no changes in CD46 expression between wk 0 and 8 in both men and women (Fig. 4). In women, there was good agreement between microarray and Q-PCR results. Assays for HSPC159, ITGB3, OLFM4, and TUBB1 confirmed that these mRNAs were up-regulated after GH treatment (Fig. 4). We were unable to validate the increase in expression of APOL6 or TBC1D25 in men. A decrease in OLFM4 expression was observed; however, this was less than the change observed by microarray (Fig. 4). Thus, validation of microarray results by Q-PCR for seven target genes was mixed with agreement in women but poor agreement in men.

Discussion

This is a novel report of the effects of systemic GH treatment on transcripts in peripheral blood in men and women. GH treatment unequivocally increased circulating IGF-I levels. However, expression changes in peripheral blood were small, with maximal effects approximately 2-fold up-regulation and down-regulation. Approximately 11% of all probes expressed were significantly regulated with more differentially regulated in women. The effects of GH in men and women were different, with only three of the top 100 up-regulated or down-regulated probes ranked by fold change being common. The effect of GH was of similar magnitude to the variation between individuals at baseline. In short, a supraphysiological dose of GH induced a robust increase in IGF-I, whereas its transcriptional effects are modest and of similar magnitude to the variation between individuals.

There are potential explanations for the modest transcriptional effects of GH. It may be that GH does not exert uniform effects on transcription in a mixed cell population, despite the evidence that several subpopulations of peripheral blood cells are GH responsive (9, 10). There may be much larger effects in particular subpopulations that are masked by underrepresentation in the total sample. It is possible that the measured endpoint reflects the net result of multiple, possibly opposing effects on the same transcript in different subpopulations. Despite the heterogeneous cell population, Eady *et al.* (21) have demonstrated that a proportion of transcripts remain remarkably constant with time and that the major variation was between individuals, a finding in agreement with ours. A recent pilot study reported that GH treatment induced

significant changes in gene expression with maximum of up to 9-fold in the peripheral blood of children with GH deficiency but none fulfilling criteria for differential expression in children with Turner's syndrome (22). This observation suggests that the transcriptome response to GH therapy in a deficiency state is far greater than that in a replete state. Variation between individuals, coupled with the weak effect of GH supplementation, indicates that using whole peripheral blood, rather than individual cell populations, may confound the ability to detect GH doping by this method.

The effects of GH on peripheral blood leukocytes were different in men and women. This was true at the probe level and at the level of the underlying biology that we have also assessed by gene set enrichment analysis (data not shown). Women are less GH responsive (6). Even though women received a larger dose of GH per kilogram body weight, there was a lesser increase in IGF-I levels (Fig. 1). Sex steroids can modulate GH action, with estrogens attenuating and androgens enhancing the biological effects of GH (23, 24). Indeed, there is evidence for a number of differential effects of sex hormones on immune responses in humans (25). Thus, it is not unexpected that we have demonstrated gender-specific effects.

A similar percentage of probes was identified as being differentially regulated in both men and women (Table 1). However, considering the results by FDR, more probes were differentially regulated in women at all cutoffs. This observation may reflect greater statistical power from 13 *vs.* seven participants in women and men, respectively, and may explain the apparently better concordance between microarray and Q-PCR data for women. From the sample of genes selected, we identified two false-positive probes in men and an additional probe with an expression change less than that predicted by microarray. All genes selected in women performed as expected. Overall, our results are representative of the small effect of GH on this system relative to the inter-individual variation in expression.

Our ranking by fold change identified six genes that were commonly and differentially regulated by GH in men and women, three of which were up-regulated (IGF-II, MED18, and PDK4) and three down-regulated (CYYR1, AREG, and ARG1). These genes are involved in growth, transcriptional regulation, differentiation, and metabolism. Unlike IGF-I, IGF-II, a potent cellular growth factor, is not known to be a GH-regulated gene. However, it is up-regulated by IGF-I in the kidneys of mice (26), suggesting the GH-induced response may have been indirect. MED18 is a component of the Mediator complex, a coactivator involved in the regulated transcription of nearly all RNA polymerase II-dependent genes, suggesting a role of GH in transcriptional activation (27). Pyruvate dehydrogenase kinase isozyme 4 (PDK4), a mitochondrial enzyme, inactivates the pyruvate dehydrogenase complex, the key enzyme converting pyruvate to acetyl-coenzyme A for oxidation in the tricarboxylic acid cycle. Thus, the effect is predicted to inhibit glucose use. We recently reported that GH treatment represses the transcription of PDK4 in muscle (28). This effect is opposite to that observed in leukocytes, suggesting that glucose metabolism may be regulated by GH in a tissue-specific manner.

Among the down-regulated transcripts, *CYYR1* is located on human chromosome 21, and its product has no similarity to any known protein and is of unknown function (29). Amphiregulin (AREG), a member of the epidermal growth factor family promotes the growth of normal epithelial cells but inhibits the growth of certain carcinoma cell lines (30). Qi *et al.* (31) have reported that amphiregulin is expressed in the basophil population of peripheral leukocytes and responds to IL-3 stimulation, suggesting a role in type 2 immune response. Amphiregulin was up-regulated by GH in peripheral leukocytes of GH-deficient children in contrast to our finding in adults (22). The reason is not known but could reflect an age-dependant effect. Arginase 1 (ARG1) is the enzyme involved in final step of the urea cycle, converting L-arginine into L-ornithine and urea in the liver. Arginase 1 has been identified in macrophages, where it converts L-arginine to nitric oxide, which enhances tumoricidal activity (32). The significance of these transcript changes in peripheral blood leukocytes is unknown.

We attempted to identify small groups of probes with diagnostic potential for GH administration in men and women. A multivariate analysis approach using SDDA identified probes that could discriminate between baseline and final treatment samples. However, on cross-validation including all technical replicates, the discrimination was not maintained. Further analyses may identify probe sets that are discriminant over a certain threshold. Although we identified a number of probes that were regulated consistently across all individuals within a treatment group, most of these probes displayed very limited fold changes. The weak effect of GH on the transcriptome of peripheral blood prevents identification of diagnostic markers of GH treatment.

In summary, the small fold changes in probe expression levels indicate that GH induces a subtle effect despite causing unequivocal increases in circulating IGF-I levels. Because GH-induced alterations in gene expression are small and of the order of variation between individuals, discriminant gene signatures cannot be defined. We conclude that gene expression analysis of peripheral blood leukocytes is unlikely to be a viable approach for the detection

of GH doping and offers no advantage to the measurement of circulating protein markers of GH abuse.

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