

## **Corticosteroids and infliximab impair the performance of interferon-gamma release assays used for diagnosis of latent tuberculosis**

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**Short title:**

Corticosteroids and infliximab impair IGRA performance

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## ABSTRACT

The impact of immunosuppression on interferon-gamma release assays and novel cytokine biomarkers of TB infection, mycobacteria-specific IL-2, IP-10 and TNF- $\alpha$  responses, was investigated in an *ex vivo* model. Cytokine responses in standard QuantiFERON-TB Gold in-Tube (QFT-GIT) assays were compared with duplicate assays containing dexamethasone or infliximab. Dexamethasone converted QFT-GIT results from positive to negative in 30% of participants. Antigen-stimulated interferon- $\gamma$ , IL-2 and TNF- $\alpha$  responses were markedly reduced, but IP-10 responses were preserved. Infliximab caused QFT-GIT result conversion in up to 30% of participants, and substantial reductions in all cytokine responses. Therefore, corticosteroids and anti-TNF- $\alpha$  agents significantly impair IGRA performance. IP-10 may be a more robust TB biomarker than interferon- $\gamma$  in patients receiving corticosteroids.

## INTRODUCTION

Drugs inhibiting TNF- $\alpha$ , a key cytokine in autoimmune and antimicrobial inflammation, are increasingly used to treat chronic inflammatory conditions, including inflammatory bowel disease and rheumatoid arthritis. However, anti-TNF- $\alpha$  agents greatly increase the risk of progression from latent tuberculosis (TB) infection (LTBI) to active TB.<sup>1</sup> Consequently, many guidelines recommend screening patients for LTBI prior to commencing anti-TNF- $\alpha$  therapy with interferon- $\gamma$  release assays (IGRA).<sup>2</sup> QuantiFERON-TB-Gold is currently the most widely used IGRA in clinical practice.<sup>3</sup>

The key issue with using IGRAs in this setting is that the majority of patients are already receiving immunosuppressive drugs when anti-TNF- $\alpha$  therapy initiation is considered.<sup>4</sup> IGRAs are functional assays based on interferon- $\gamma$  production by T-cells in response to stimulation with TB-specific antigens *in vitro*.<sup>5</sup> Reduction in T-cell numbers (e.g. in HIV-infection) results in impaired IGRA performance.<sup>6</sup> Thus, any functional impairment of T-cells, including iatrogenic immunosuppression, will likely impact IGRA performance.

Several clinical studies have investigated IGRAs for LTBI diagnosis in immunosuppressed patients.<sup>7, 8</sup> However, all have one significant limitation - the lack of a gold-standard to compare test performance against. In contrast to active TB, where microbiological detection of *Mycobacterium tuberculosis* (Mtb) remains the gold-standard, no such standard exists for LTBI.<sup>5</sup> Therefore, interpretation of IGRAs in immunosuppressed patients is complex, as it is impossible to determine whether a

negative result reflects absence of TB infection or alternatively a false-negative result caused by immunosuppressive agents.

A recent meta-analysis further supports immunosuppressive drugs interfering with IGRAs.<sup>9</sup> Patients receiving corticosteroids or anti-TNF- $\alpha$  agents had significantly lower rates of positive IGRA results than those not treated with these agents.

However, this meta-analysis had limitations, primarily related to insufficient data included in the original reports. Importantly, many lacked data on concomitant medication, and therefore this meta-analysis may have overestimated the effect of individual drugs.

We aimed to determine the impact of corticosteroids and infliximab on the performance of QuantiFERON-TB Gold In-Tube (QFT-GIT) assays using an *ex vivo* model. Additionally, the effect of these immunosuppressive agents on mycobacteria-specific IL-2, IP-10 and TNF- $\alpha$  responses, recently identified biomarkers of TB infection, was investigated.<sup>10, 11</sup>

## **METHODS**

Patients with a previous positive IGRA result or recent TB contact were recruited following informed consent. Potential participants with known immunodeficiency, receiving immunosuppressive medication, or with uncontrolled diabetes mellitus were excluded.

From each participant, four QFT-GIT assay sets (Cellestis/Qiagen, Carnegie, Australia) comprising an antigen stimulated, a positive control and a negative control sample were obtained. In the first set, no reagents were added ('standard assay'). In the second set, dexamethasone (Organon Laboratories, Cambridge, UK) was added to each tube at 2µg/mL, based on pharmacokinetic data.<sup>12</sup> In the third and fourth sets, infliximab (MSD, Hoddesdon, UK) was added to each tube at 5µg/mL ('low-dose') and 100µg/mL ('high-dose'), respectively, corresponding to therapeutic trough and peak levels.<sup>13</sup> Samples were then incubated within 4 hours of phlebotomy at 37°C for 24 hours, as per manufacturer's instructions. Supernatants were then harvested and cryopreserved for batched analysis.

Cytokine concentrations were determined with ProcartaPlex xMAP-assays (eBioscience, Hatfield, UK) measuring interferon- $\gamma$ , IP-10, IL-2 and TNF- $\alpha$  following the manufacturer's instruction. Their broad dynamic range allows accurate measurement of high interferon- $\gamma$  concentrations that frequently occur in QFT-GIT samples, exceeding the upper limit of QFT-GIT ELISAs.<sup>14</sup> Cytokine concentrations were measured using a Luminex 100 Bioanalyzer (Luminex, Austin, U.S.). QFT-GIT results were interpreted according to the manufacturer's package insert (latest U.K. version).<sup>15</sup>

Interferon- $\gamma$  concentrations measured in pg/mL were converted to IU/mL (the units used in QFT-GIT), as previously described.<sup>16</sup> All other cytokines were analysed in pg/mL. Statistical analyses were performed in Prism (V5.0; GraphPad, La Jolla, U.S.). Wilcoxon matched-pairs signed-rank tests were used to compare cytokine concentrations in standard assays with those in assays with added drugs.

## **RESULTS**

Nineteen adults (12 male; 7 female), median age 45 (range: 20-68) years, were recruited. None of the participants had been started on anti-TB treatment prior to recruitment. HIV testing was performed in 16 participants (three declined testing); all had negative test results. Ten participants had positive QFT-GIT results in the standard assay, while nine were negative. Consequently, for analyses of antigen-stimulated cytokine responses only data from the former ten participants were included, while data from all 19 were included in analyses related to mitogen (positive control) responses.

### **Interferon- $\gamma$ responses and categorical QFT-GIT results**

Of the ten participants with positive QFT-GIT results in the standard assay, three had negative QFT-GIT results in the set with added dexamethasone, while seven remained positive. In the set with low-dose infliximab, two had negative QFT-GIT results, and eight remained positive. In the set with high-dose infliximab, two had negative QFT-GIT results and one an indeterminate result; in seven the result remained positive.

Compared with the standard assay, the background-corrected interferon- $\gamma$  concentrations in antigen-stimulated samples were significantly lower with all added drugs (Figure 1). Dexamethasone resulted in a 4.8-fold reduction; low-dose infliximab and high-dose infliximab resulted in 8.5-fold and 16.4-fold reductions, respectively (Table 1). In each participant, background-corrected interferon- $\gamma$  concentrations in the antigen-stimulated samples were universally lower with added

dexamethasone or infliximab (both low- and high-dose) than in the standard assay (Supplementary Figure 1).

In the positive control samples, dexamethasone caused a significant reduction in the background-corrected interferon- $\gamma$  concentrations; in contrast, infliximab had no significant impact on positive control responses (Figure 1).

### **IL-2, IP-10 and TNF- $\alpha$ responses**

Dexamethasone resulted in reduced background-corrected IL-2 and TNF- $\alpha$  concentrations in antigen-stimulated samples (Figure 1). Although the average reduction of both cytokines was substantial, this was only statistically significant for IL-2 (Table 1). In contrast, dexamethasone had no substantial impact on background-corrected IP-10 concentrations in antigen-stimulated and positive control samples (Figure 1 & Table 1). Both low-dose and high-dose infliximab resulted in a significant reduction of background-corrected IL-2, IP-10 and TNF- $\alpha$  concentrations in antigen-stimulated samples (Figure 1 & Table 1).

## **DISCUSSION**

To our knowledge, this is the first study to use an *ex vivo* model to determine the impact of corticosteroids and infliximab on the performance of QFT-GIT assays. Importantly, this approach overcomes the key limitation of previous clinical studies that were hindered by the absence of a gold standard for LTBI.

We show that corticosteroids impair the performance of QFT-GIT assays significantly. On average, antigen-stimulated interferon- $\gamma$  responses were almost 5-fold lower with dexamethasone compared to the standard assays. Our finding that dexamethasone at therapeutic concentrations led to QFT-GIT assay results changing from positive to negative in 30% of participants is consistent with our recent study that investigated the impact of anti-tuberculous antibiotics and corticosteroids on QFT-GIT assays, where result conversion with dexamethasone occurred in four of ten participants.<sup>17</sup> The observation that dexamethasone also reduces positive control responses in QFT-GIT assays may explain the comparatively high proportions of indeterminate QFT-GIT results observed in patients receiving oral corticosteroids in previous clinical studies.<sup>18</sup> These findings are consistent with *in vitro* studies showing that corticosteroids inhibit pro-inflammatory cytokine production from T-cells, including interferon- $\gamma$ .<sup>19</sup>

Our observations are highly relevant to clinical practice, as they show that IGRAs may be unreliable in patients receiving corticosteroids. Therefore, the recommendation to screen patients on ‘conventional’ therapy with IGRAs before starting biological therapy, as suggested by most guidelines, is flawed. Based on first principles it is probable that other immunosuppressive agents targeting T-cell function, including calcineurin inhibitors and purine analogues, also impair IGRA performance. Consequently, guidelines should be amended so that IGRA testing is performed when a patient is first diagnosed with a disease that may ultimately require treatment with biological agents, rather than after initiation of immunosuppressive treatment. In patients already receiving immunosuppressive drugs, we and other experts recommend that IGRAs are used in conjunction with TSTs to increase

diagnostic yield, as detailed elsewhere.<sup>2, 20</sup> Furthermore, patients with a negative IGRA result at diagnosis and subsequent TB exposure, or at risk of exposure due to travel to or residence in a high TB prevalence, should be re-screened prior to starting biological agents.

Our results show that in the presence of corticosteroids IP-10 responses were sustained, indicating that IP-10 may be a more robust marker of TB infection in patients receiving corticosteroids. The underlying mechanism for this observation remains uncertain. However, in contrast to interferon- $\gamma$ , IP-10 is mainly produced by monocytes, endothelial cells and fibroblasts and there are data suggesting that corticosteroids have only limited impact on intracellular inflammatory signaling in monocytes.<sup>21</sup> Several recent studies, including our own, highlight the potential of Mtb-specific IP-10 responses as novel biomarkers for the diagnosis of both LTBI and active TB in immunocompetent patients.<sup>10, 11</sup> One small study in immunocompromised patients with active TB reported that Mtb-specific IP-10 responses did not differ significantly from those in active TB patients without immunocompromise, further supporting the notion that IP-10 may be a better biomarker than interferon- $\gamma$  in immunocompromised individuals.<sup>11</sup>

Our data also suggest that the performance of IGRAs is significantly impaired by infliximab. We observed a considerable reduction in Mtb-specific interferon- $\gamma$  responses, and changes in categorical results in up to 30% of patients. These observations are concordant with current concepts of human anti-mycobacterial immune responses. TNF- $\alpha$ , produced by antigen-presenting cells (APCs), plays a critical role in T-cell/APC interactions by aiding granuloma formation and stimulating

Mtb-specific T-cells to produce interferon- $\gamma$ .<sup>22</sup> Interferon- $\gamma$  stimulates TNF- $\alpha$  production in APCs, thereby closing the feedback-loop. Anti-TNF- $\alpha$  agents interrupt this interaction, thereby diminishing the ability to contain mycobacteria, and reducing the ability to generate adequate anti-mycobacterial responses in functional assays, such as IGRAs. The latter is further supported by the observation that infliximab markedly reduced all four Mtb-specific cytokine responses investigated.

In conclusion, we provide evidence that corticosteroids and infliximab significantly impair QFT-GIT performance. A substantial proportion of patients with LTBI receiving either drug are likely to have false-negative results if tested with this IGRA. Therefore, LTBI screening should be performed at time of diagnosis, not after the initiation of immunosuppressive treatment. Mtb-specific IP-10 responses may be a more robust marker of LTBI in patients receiving corticosteroids than interferon- $\gamma$  responses, which form the basis of commercial IGRAs.

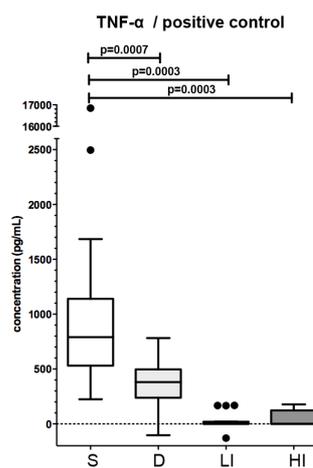
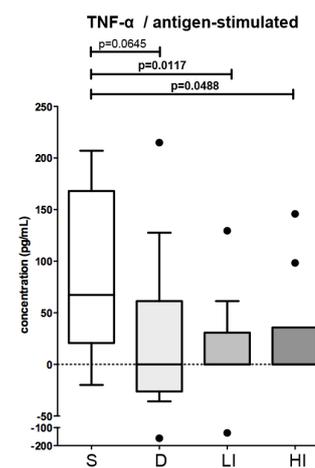
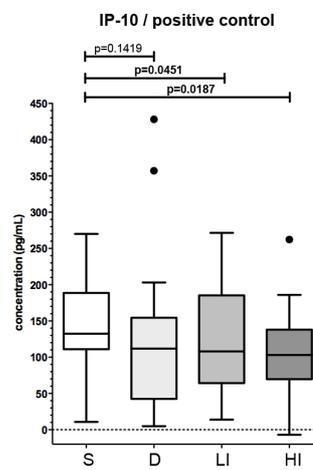
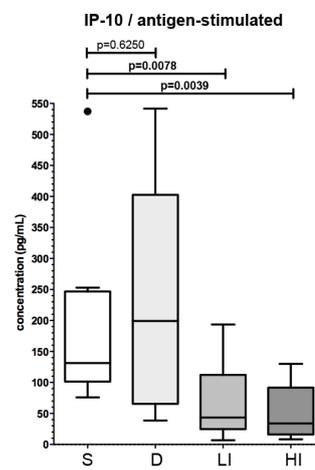
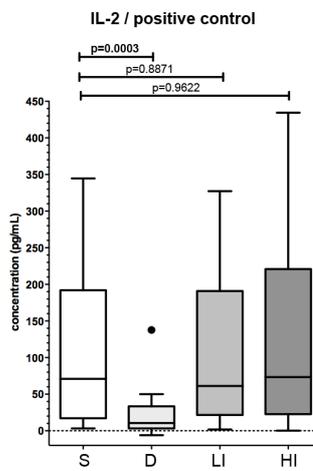
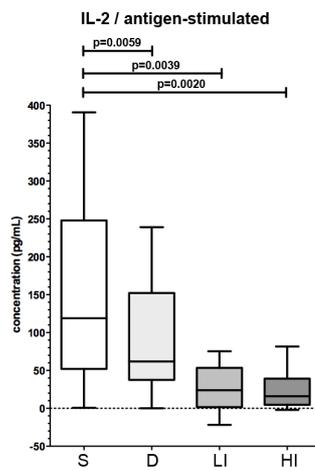
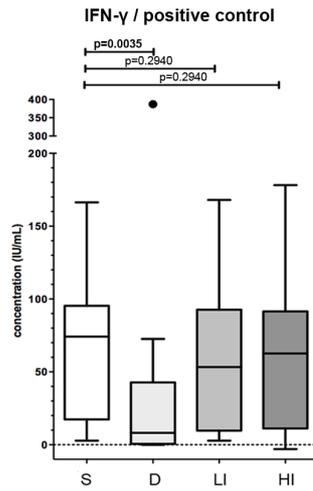
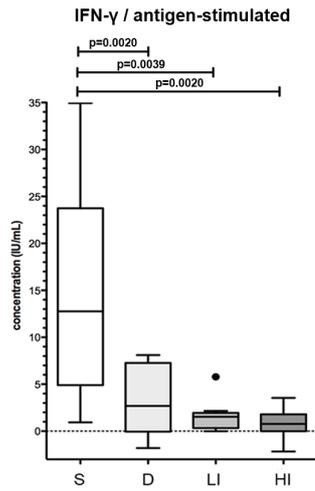
**Contributors:** Study concept and design: AE, YG, RNA, VC, NC, AW, PE, and MT; participant recruitment: BM, PE, and MT; sample processing and analysis: AE, YG, DB, and MT; analysis of data: AE, YG, RNA, HdG, TC, VC, NC, PE, and MT; data interpretation and drafting of the manuscript: AE, VC, NC, SM, PE, and MT. All authors critically read, commented on, and approved the final version of the manuscript.

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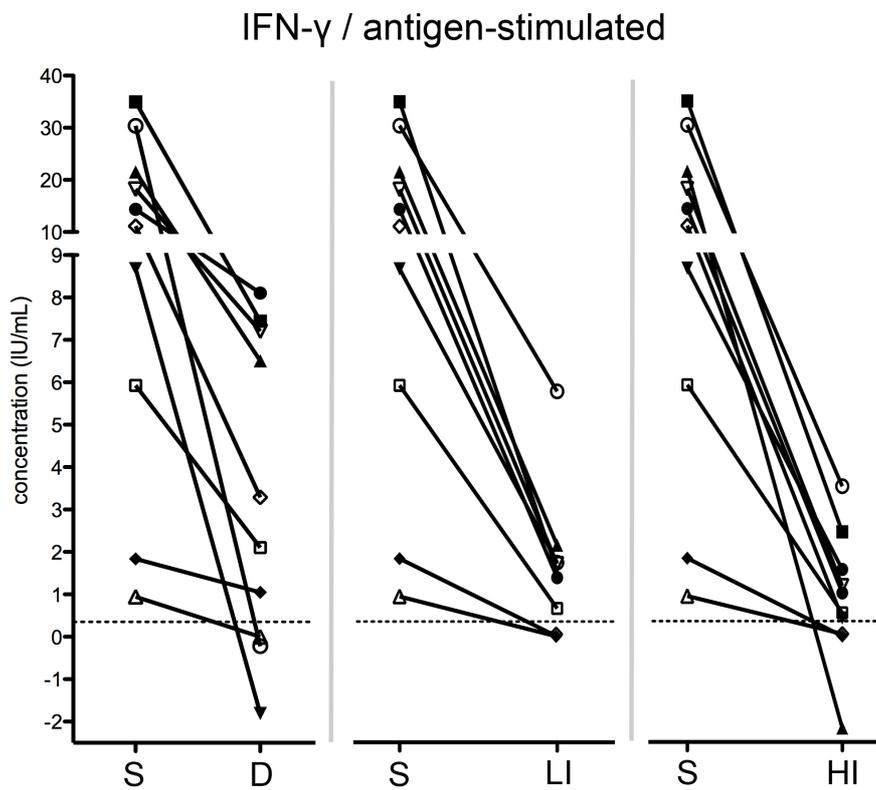
**Competing interest:** MT has received QuantiFERON-TB Gold assays at reduced cost for related research projects from the manufacturer (Cellestis/Qiagen). The manufacturer had no influence on the study design, data interpretation, writing of the manuscript or decision to submit the data for publication.

**Ethics approval:** The study was approved by the National Research Ethics Service Committee South Central (approval number 13/SC/0043).

**Figure 1. Antigen-stimulated and positive control interferon- $\gamma$ , IL-2, IP-10 and TNF- $\alpha$  responses.** Box plot with Tukey whiskers showing background-corrected cytokine concentrations in antigen-stimulated (left) and positive control (right) samples in standard (S) QFT-GIT assays (no additive) and those with added dexamethasone (D), low-dose infliximab (LI) and high-dose infliximab (HI). The horizontal lines depict the medians. The p-values were calculated with two-tailed Wilcoxon matched-pairs signed-rank tests.



**Supplementary Figure 1. Background-corrected interferon- $\gamma$  responses in antigen-stimulated samples** in standard (S) QFT-GIT assays (no additive) and those with added dexamethasone (D; left panel), low-dose infliximab (LI; middle panel) and high-dose infliximab (HI; right panel). The dotted line indicates the QFT-GIT cut-off for assay positivity (0.35 IU/mL).



**Table 1. Background-corrected cytokine concentrations in antigen-stimulated QFT-GIT samples.** Only data from participants with a positive QFT-GIT result in the standard assay were included in this analysis (n=10).

<b>Cytokine</b>	<b>Standard assay median conc. (IQR) *</b>	<b>Dexamethasone median conc. (IQR) *</b>	<b>Dexamethasone ratio **</b>	<b>Low-dose infliximab median conc. (IQR) *</b>	<b>Low-dose infliximab ratio **</b>	<b>High-dose infliximab median conc. (IQR) *</b>	<b>High-dose infliximab ratio **</b>
<b>IFN-<math>\gamma</math></b>	12.8 (4.9 - 23.7)	2.7 (-0.1 - 7.3)	-4.8	1.5 (0.33 - 1.9)	-8.5	0.8 (-2.1 - 1.8)	-16.4
<b>IL-2</b>	118.9 (52.0 - 247.9)	61.9 (37.6 - 152.3)	-1.9	23.9 (1.6 - 53.4)	-5.0	16.1 (4.7 - 39.2)	-7.4
<b>IP-10</b>	131.4 (101.3 - 246.7)	199.2 (65.6 - 402.7)	+0.7	43.4 (24.8 - 112.2)	-3.0	33.9 (16.1 - 91.7)	-3.9
<b>TNF-<math>\alpha</math></b>	67.3 (20.8 - 168.0)	0.0 (-26.2 - 61.3)	- ***	0.0 (0.0 - 30.8)	- ***	0.0 (0.0 - 145.8)	- ***

\*Cytokine concentrations in pg/mL, except interferon- $\gamma$  concentrations (IU/mL).

\*\*Ratios were calculated by dividing median background-corrected cytokine concentrations in standard assays by median background-corrected cytokine concentrations in assays with added drugs.

\*\*\*Value cannot be calculated as one of the cells contains a zero value.

Abbreviations: conc.: concentration; IFN- $\gamma$ : interferon- $\gamma$ ; IQR: interquartile range.

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