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## Mutations in the signal transducer and activator of transcription 3 (STAT3) and diagnostic guidelines for the Hyper-IgE Syndrome

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### Clinical Implications

These diagnostic guidelines for STAT3-deficient HIES will facilitate time- and resource-saving diagnosis of the patients, thereby leading to an early and effective treatment.

### Capsule Summary

The diagnostic guidelines for STAT3-deficient HIES will aid the diagnostic process of HIES patients.

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## Abstract

**Background**—The hyper-IgE syndrome (HIES) is a primary immunodeficiency characterized by infections of the lung and skin, elevated serum IgE, and involvement of the soft and tissues. Recently, HIES has been associated with heterozygous dominant-negative mutations in *STAT3* and severe reductions of Th17 cells.

**Objective**—To determine whether there is a correlation between the genotype and phenotype of HIES patients and to establish diagnostic criteria to distinguish between *STAT3* mutated and *STAT3* wild-type patients.

**Methods**—We collected clinical data, determined Th17 cell numbers, and sequenced *STAT3* 100 patients with a strong clinical suspicion of HIES and serum IgE >1000 IU/mL. explored diagnostic criteria by using a machine-learning approach to identify which features best predict a *STAT3* mutation.

**Results**—In 64 patients we identified 31 different *STAT3* mutations, 18 of which are novel. These included mutations at splice sites and outside the previously implicated DNA-binding and SH2 domains. A combination of five clinical features predicted *STAT3* mutations with 85% accuracy. Th17 cells were profoundly reduced in patients harboring *STAT3* mutations, while 10 out of 13 patients without mutations had low (<1%) Th17 cells but were distinct markedly reduced IFN- $\gamma$  producing CD4<sup>+</sup> T cells.

**Conclusions**—We propose the following diagnostic guidelines for *STAT3*-deficient HIES: Possible: IgE >1000 IU/mL plus a weighted score of clinical features >30 based on recurrent pneumonia, newborn rash, pathologic bone fractures, characteristic face, and high palate. Probable: Above plus lack of Th17 cells or a family history for definitive HIES. Definitive: Above plus a dominant-negative heterozygous mutation in *STAT3*.

## Keywords

Hyper-IgE Syndrome; HIES; Job syndrome; Th17 cells; *STAT3*-mutations; diagnostic guidelines

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## INTRODUCTION

The hyper IgE syndrome (HIES) is a multisystem disorder characterized by eczema, skin abscesses, recurrent staphylococcal infections of the skin and lungs, pneumatocele formation, candidiasis, eosinophilia, and elevated serum levels of IgE. Non-immunologic features of HIES include characteristic facial appearance, scoliosis, retained primary teeth, joint hyperextensibility, bone fractures following minimal trauma, and craniosynostosis.<sup>1–3</sup> In 1999, the NIH clinical HIES scoring system based on 19 clinical and laboratory findings was introduced.<sup>4</sup> A point scale was developed: more specific and objective findings were assigned more points. Scores of at least 40 points suggested HIES, while a score of below 20 made the diagnosis unlikely. For intermediate values, no firm conclusion could be reached.

In 2007, mutations in the signal transducer and activator of transcription 3 (*STAT3*) were shown to be a cause of HIES.<sup>5,6</sup> *STAT3* plays a key role in the signal transduction of a broad range of cytokines.<sup>7,8</sup> After cytokine binding and JAK (Janus kinase) activation, *STAT3* is phosphorylated, dimerizes and translocates to the nucleus, where it controls transcription of its target genes.<sup>9</sup> *STAT3* is crucial for the IL-6-mediated regulation of Th17 cells that are significant producers of IL-17, a proinflammatory cytokine involved in the host defense of *S. aureus* and *Candida*.<sup>10–12</sup>

There are seven publications on *STAT3* mutations reporting on 155 patients with HIES. In 141 of these patients, heterozygous mutations in *STAT3* were identified.<sup>5,6,13–17</sup> Therefore, we addressed the question: how common is a diagnosis of HIES without a *STAT3* mutation? We also asked: do some features of the HIES phenotype make it more likely that an HIES patient has a *STAT3* mutation and can any feature(s) of the HIES phenotype predict the location of mutations within *STAT3*; i.e., is there any phenotype-genotype correlation?

As *STAT3* has 24 exons and three splice variants, predicting which patients are likely to have a *STAT3* mutation could save sequencing resources.

In a multi-center cohort of 100 patients with suspected HIES, we evaluated 17 of the clinical and laboratory features used in the original scoring method,<sup>4</sup> the recently reported laboratory feature of a very low Th17 CD4<sup>+</sup> T cell count, and the genetic diagnosis to develop a new scoring system aimed to discern those HIES patients with *STAT3* mutations from those without mutation.<sup>14–16</sup>

Based on our analysis of 100 unrelated patients, evaluated world-wide, we propose guidelines for a clinical assessment prior to a confirmation of the suspected diagnosis by laboratory and molecular analysis.

## METHODS

### Patients and controls

Over the last eight years, we have collected a cohort of 228 patients with the suspected diagnosis of HIES in a world-wide collaboration; 55 of these patients have been published elsewhere.<sup>6,13,17,18</sup> Of the remaining patients, 100 *unrelated* patients fulfilled inclusion criteria for this study: signed consent form, complete NIH scoring sheet, a strong clinical suspicion of HIES according to the referring immunologist, an IgE >1000 IU/mL, and an available sample of

genomic DNA (gDNA) or RNA. To promote uniformity of documentation across the 33 different participating centers, a scoring sheet listing the original NIH clinical symptoms was used.<sup>4</sup>

Of the 100 patients with the clinical suspicion of HIES, 61 were male and 39 female; the age of the patients at the time of clinical evaluation ranged between 1 and 58 years. Seventy-two patients came from Europe, 20 from the Middle East, seven from South America, and one from North America.

Eighty patients had HIES scores  $\geq 40$  suggesting that these patients probably had HIES, whereas 20 patients had scores below 40, suggesting a diagnostic uncertainty or a variant of HIES.<sup>19</sup> Only two of these patients (UPN133 and 134) have been described in published *STAT3* mutation reports.<sup>17</sup> Detailed information on patients, including clinical scores and detected *STAT3* mutations, are summarized in Table E1 and in reference<sup>20</sup>.

We applied the diagnostic guidelines, developed using the clinical scores of the cohort of 100 patients to a replication set of 50 unrelated patients all scored by a consistent team of clinicians at the NIH. Of these 50, 33 had a mutation in *STAT3* and 17 did not; the 33 patients with a *STAT3* mutation were from a previously published cohort.<sup>6</sup> In addition 28 patients with severe atopic dermatitis and an IgE level  $>1000$ IU/ml were scored.

Control DNA was isolated from 100 healthy Caucasians according to approved protocols. *STAT3* was sequenced in all controls to verify that the sequence changes seen in patients were not frequent polymorphisms. In addition, twelve controls were studied for their lymphocyte phenotype. All patients and controls or their parental or legal guardians provided written consent for the conducted studies, following local ethics committee requirements.

### PCR and Sequencing

Genomic DNA and RNA of controls and patients was isolated from either whole blood or peripheral blood mononuclear cells (PBMCs) using RNeasy Kit (Qiagen) according to manufacturer's instructions. RNA was reverse transcribed using Omniscript reverse transcriptase (Qiagen).

Coding genomic sequences and cDNA of *STAT3* were amplified and purified using the QIAquick PCR purification kit (Qiagen). Primer sequences are available upon request. Purified PCR products were sequenced with the ABI PRISM BigDye Terminator cycle ready reaction kit V3.1 (Applied Biosystems, Foster City, CA) using the PCR primers as sequencing primers. The sequencing was performed on a 3130xl Applied Biosystems Genetic Analyzer, and the data were analyzed with DNA Sequencing Analysis software, version 5.2 (Applied Biosystems) and Sequencher™ version 4.8 (Gene Codes Corporation, Ann Arbor, USA).

### Cell culture and TNF- $\alpha$ ELISA

Monocyte-derived macrophages were generated as previously described,<sup>21</sup> kept in Opti-Mem I serum free medium (Invitrogen, UK), and differentiated by using 50 ng/mL M-CSF (PeproTech, UK). After five days of culture, the monocytes/macrophages were pre-incubated with 25 ng/mL IL-10 (R&D) for one hour and then stimulated overnight with 50 ng/mL *E.coli* LPS (Sigma). TNF- $\alpha$  release was measured by ELISA (PeproTech) according to manufacturer's instructions.

### Lymphocyte stimulation and Flow Cytometry

Blood was collected from 30 patients and 12 healthy donors. PBMCs were isolated using Lymphoprep™ (Axis-Shield) and viably frozen. Freshly thawed cells resuspended at a

concentration of  $10^6$ /mL in RPMI (Lonza), supplemented with 10% fetal calf serum (FCS, Gibco), were exposed to Staphylococcus enterotoxin B (SEB; Sigma)  $1\mu\text{g}/\text{mL}$  and Brefeldin A (Sigma)  $2.5\mu\text{g}/\text{mL}$  16 hours at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . The cells were then surface-stained for CD4 (PerCP) and CD45RO (PE-Cy7), followed by an intracellular staining for IL-17 (Alexa Fluor<sup>®</sup> 647) and IFN- $\gamma$  (FITC). Intracellular staining was performed by using the BD Cytotfix/Cytoperm<sup>™</sup> Fixation/Permeabilization Kit (BD Biosciences). All antibodies were from BD Biosciences, except for IL-17 which was from eBioscience. Data were acquired on an LSR II flow cytometer and analyzed using FACSDiva software (BD Biosciences). Wilcoxon rank-sum tests were performed with Prism 5.01 software (Prism); P-values are two-sided.

### Statistical Analysis

Univariate statistical analyses were done using GNU R.<sup>22</sup> For tests where we had a prior hypothesis (e.g., higher clinical score is associated with a *STAT3* mutation), P-values were one-sided; otherwise P-values were two-sided. A Bonferroni correction was applied to each series of univariate tests.

The 100 patients in the cohort form our data set for univariate analysis and our training set for the machine learning methods described below. The “gold standard” for the diagnosis of *STAT3*-deficient HIES was the discovery of a heterozygous mutation (most mutations proven to be dominant negative) in *STAT3* using the DNA sequencing techniques described above. Table I shows the prevalence of each feature in our HIES cohort. Based on preliminary experiments, we found that support vector machines (SVMs) are an effective way to classify this data set. To compare feature sets,<sup>23</sup> we took the scores for 17 of the 19 features<sup>4</sup> and calculated the leave-one-out accuracy for SVMs generated from each subset of size at most two and no more than seven. Because there are 41,208 such subsets, we used the software package OOQP (<http://pages.cs.wisc.edu/~swright/ooqp/>),<sup>24</sup> which contains specialized code to generate linear SVMs. We used a pre-release version of OOQP (available from E.M.G.) that provides leave-one-out estimation and that has been optimized for speed.

We chose as candidate feature sets those 344 sets for which OOQP reported leave-one-out accuracy of better than 80%. Leave-one-out testing was performed with OOQP and SVMlight (<http://svmlight.joachims.org/>),<sup>25</sup> as described in the Online Repository. For several feature sets, a classifier was trained using SVMlight on the scores from our cohort of 100 patients and then applied to predict whether each of the patients in the replication set has a *STAT3* mutation.

## RESULTS

### STAT3 mutations

Of the 100 unrelated patients with suspected HIES, 64 carried heterozygous mutations within *STAT3*. Thirty-six patients did not show any mutation in the coding regions of *STAT3* or their flanking intronic sequences. Overall, we found 31 distinct mutations: 46 patients carried previously described mutations,<sup>5,6,13–17</sup> and 18 patients harbored distinct, novel mutations. Of these 18 mutations, eight affected the DNA-binding domain, five the SH2 domain, four the transactivation domain and one the coiled-coil domain (see Table II).

Five mutations, seen in six patients, were heterozygous mutations at the intron-exon boundaries of exons 12 or 22, affecting the DNA binding and transactivation domains, respectively. Each of these intronic mutations led to an incorrect splicing of its neighboring exon (data not shown). Three patients had two distinct heterozygous mutations at the 3' splice site prior to exon 12, and two patients had distinct mutations in at the 5' splice site following exon 12. Two of these four mutations were described by Renner et al the other two are novel.<sup>13</sup> All four mutations cause exon 12 to be skipped, suggesting an in-frame deletion of amino acids 371–380.



None of 100 healthy controls carried any of the mutations listed in Table II.

To analyze the effects of the newly observed *STAT3* mutation *in vitro*, we used a TNF- $\alpha$  release assay as described previously by Minegishi et al.<sup>5</sup> We pre-incubated monocytes/macrophages of ten patients and eleven healthy controls with IL-10 for one hour and then stimulated with LPS. Eight of the ten patients tested had novel mutations, one was wild-type for *STAT3*, and one had the previously-reported mutation R382Q. In healthy controls and a *STAT3* wild-type patient, IL-10 reduced TNF- $\alpha$  release by approximately 90%, whereas in the R382Q patient, TNF- $\alpha$  release was only reduced by 40%. All eight novel mutations showed a reduction in IL-10-mediated down regulation of TNF- $\alpha$  secretion. We compared the effect of the eight novel mutations collectively to eleven healthy controls by a rank-sum test and reached significance ( $p < 0.0001$ ). However not all mutations had the same effect, suggesting that some mutations may lead to *STAT3* proteins with a hypomorphic function and possibly to a milder phenotype (see Figure 1).

A substantial portion (36 patients) of our cohort did *not* have mutations in the coding or splice regions of *STAT3*. To exclude undetected splice site variants not described in healthy individuals, we sequenced the cDNA from 25 of these patients, none of whom revealed any mutation or exon skipping. Due to the limited availability of samples, the cDNA of eleven patients could not be tested (UPN 21, 28, 35, 38, 58, 78, 80, 82, 88, 105 and 112). While some patients lacking *STAT3* mutations also had NIH HIES scores below the likely diagnostic cutoff of 40 points, 20 (56%) had scores  $\geq 40$  (see Table E1).

### Evaluating the value of the NIH-HIES clinical scoring system

To evaluate the NIH-HIES scoring system,<sup>4</sup> we performed three Wilcoxon rank-sum tests to calculate the significance of the association of total clinical HIES score with mutation status. High total HIES score was strongly associated with having a mutation in *STAT3* (P-value  $3.9 \times 10^{-7}$ ). Among those patients with a *STAT3* mutation, a high total HIES score may carry some information about whether the mutation is located in the DNA binding domain (P-value 0.094) but was not significantly associated with having a mutation in the SH2 domain, rather than elsewhere in *STAT3* (Table E2).

We evaluated whether any cut-off value for the NIH HIES score would be useful for predicting whether a patient has a *STAT3* mutation. Using the rule “choose the largest threshold T that gives the smallest error”, leave-one-out testing gave 23% error, 75% sensitivity, and 80.1% selectivity. The optimal cutoff was  $T=49.5$ . On the replication set of 50 patients, the cutoff  $T=49.5$  has a sensitivity of 97.0%, but a selectivity of 58.8%. Therefore we sought a smaller and more precise set of features than the NIH HIES score. These seemingly competing objectives could be achieved with a linear support vector machine.

### Set of clinical features best predicting the genotype

In search of a minimal set of clinical features associated with *STAT3* mutation-positive HIES, we prospectively scored the 100 patients according to the NIH HIES method,<sup>4</sup> and performed leave-one-out error estimates for all feature sets tested.

There were twelve sets with a leave-one-out accuracy of 85% (Table E3). By logistic regression of the individual features occurring in at least two feature sets, pneumonia ( $P = 0.002$ ), lung cysts ( $P = 0.001$ ) and characteristic face ( $P = 0.002$ ) were positively associated with a *STAT3* mutation. Of the twelve sets one had five features, all other sets had more than five features. These five features are i) pneumonia, ii) newborn rash, iii) pathologic fractures, iv) characteristic face of Job syndrome and v) cathedral palate. Notably, a HIES patient does not need to have severe scores for all five features to get a total score above the threshold of 30

points for predicting a *STAT3* mutation (Table E4). Detailed information about the five chosen features is summarized in the Online Repository.

Although some clinical features may take years to develop, the misclassification rate on young children (5/37 for age 11 and younger; 2/17 for age 7 and younger) was comparable to those of the entire cohort (see Table E5). Therefore, this set of diagnostic features can also be applied to children suspected of mutations in *STAT3*.

We applied the score on a control cohort of 28 patients with atopic dermatitis and IgE levels of >1000IU/ml and all had a weighted score of <30 points.

In leave-one-out testing, this set of clinical features in our cohort of suspected HIES patients had a sensitivity of 87.5% for predicting presence of a *STAT3* mutation, a specificity of 80.6% and an error rate of 15%. On the replication set of 50 patients, this same set of features had a sensitivity of 93.9%, a specificity of 70.6%, and an error rate of 14%. Increasing the number of features did not improve the quality of our model.

None of the eleven features shown in Table E3 was significantly associated with the domain in which the *STAT3* mutation was located, hence no phenotype-genotype correlation can be reported.

### Using Th17 cells in HIES as a diagnostic marker - IL17 and IFN- $\gamma$ production by CD4<sup>+</sup> T cells in HIES

The induction of Th17 cells following stimulation with either the superantigen SEB or the mitogen PMA has been reported to be impaired in HIES patients with *STAT3* mutations.<sup>13–17</sup> We analyzed the level of Th17 cells among the PBMCs in 30 of our 100 HIES patients and in 12 control subjects. Seventeen of these patients had mutations in *STAT3*, and 13 did not. Thirteen of the 17 patients harboring mutations in *STAT3* had less than 0.5% of IL-17 producing CD4<sup>+</sup> T cells. In contrast, all but one of our healthy donors had a frequency of more than 1%. The 13 patients without mutations in *STAT3* had significantly more IL-17-producing T cells than the *STAT3*-deficient patients ( $P = 0.003$ ), but significantly fewer than the healthy controls ( $P = 0.0001$ ); only three of these 13 patients had an extremely low frequency of IL-17-producing T cells, less than 0.5% (see Figure 2). No correlation between the lack of Th17 cells, the incidence of *Candida* infections, or the gender of the patients has been observed (see Table III).

We measured the frequency of SEB-induced interferon (IFN)- $\gamma$  producing CD4<sup>+</sup> T cells from PBMCs of the same set of subjects (see Figure 2). Interestingly, the patients without a *STAT3* mutation had significantly fewer interferon (IFN)- $\gamma$  producing CD4<sup>+</sup> T cells than did healthy controls ( $P = 0.0001$ ). Of the 13 patients lacking *STAT3* mutations, only one had >5% (IFN)- $\gamma$  producing CD4<sup>+</sup> T cells. HIES patients carrying *STAT3* mutations also had significantly lower frequencies of interferon (IFN)- $\gamma$  producing CD4<sup>+</sup> T cells than healthy controls ( $P = 0.02$ ), but significantly higher frequencies than patients without *STAT3* mutation ( $P = 0.007$ ).

We conclude that the lack of Th17 cells is a useful predictive marker for heterozygous mutations in *STAT3*.

## DISCUSSION

In our cohort of 100 patients with suspected HIES, we found 18 novel mutations in *STAT3*. As these were spread over the whole gene, it appears necessary to sequence the entire *STAT3* gene to exclude a possible mutation, an expensive process due to the size of the gene (see Figure 3).

The relative frequencies of mutations in Table II however, suggest that a cost-conscious strategy would be to sequence either cDNA or gDNA of *STAT3* in the following order: 1) the exons and intron junctions of the DNA-binding domain, 2) the exons and intron junctions of the SH2 domain 3) the exons and intron junctions of the transactivation domain and 4) the rest of the coding sequences. In all HIES patients with detected *STAT3* mutations, only one allele was affected, consistent with the observation that complete loss of *STAT3* leads to early embryonic death in *STAT3* knock-out mice.<sup>26</sup>

Mutations in the promoter region are possible, but are unlikely to cause HIES when present in heterozygosity because they will not exert a dominant-negative effect which seems necessary for the HIES phenotype.<sup>5</sup>

Although over 90% of the mutations we found are located in the exons of *STAT3*, we found five mutations at the intron-exon boundaries. Four of these mutations caused an in-frame deletion of the 10 amino acids encoded by exon 12. The effect of the splice site mutation on exon 22 is more complex. In healthy individuals, alternative splicing of exon 23 results in two major isoforms of *STAT3*: *STAT3*alpha and *STAT3*beta. While *STAT3*alpha contains the entire exon 23, *STAT3*beta lacks the first 50 bp of the same exon.<sup>27</sup> The splice site introduced by the mutation one nucleotide after exon 22 causes the 43 bp exon to be skipped. In the wild-type *STAT3*alpha transcript, this deletion would result in a frame shift and possibly nonsense mediated decay. However, in conjunction with the shortened exon 23 of *STAT3*beta, this deletion is predicted to produce a transcript coding for *STAT3*alpha with an in-frame deletion of amino-acids 701–732, which would encompass both the tyrosine and serine phosphorylation sites of *STAT3*alpha (see Figure E1).

All eight novel mutations that we tested deteriorated the suppression of LPS-induced production of TNF- $\alpha$  by the IL-10/JAK/*STAT3* pathway in the patients' macrophages. Compared to the most prevalent mutation affecting the DNA-binding domain of *STAT3* (R382Q), this effect was less prominent in some of the novel mutations. This result could reflect the fact that some mutations have a different impact on the retained functionality of the *STAT3* dimerization, nuclear translocation, or transcriptional activation.

We confirm that HIES patients carrying *STAT3* mutations have significantly reduced numbers of IL-17 producing CD4<sup>+</sup> T cells. Based on these data, we suggest that Th17 cells may be used as an additional marker to distinguish between HIES patients with or without *STAT3* mutations.

Patients without *STAT3* mutations had a striking reduction of IFN- $\gamma$  producing CD4<sup>+</sup> T cells. This cytokine imbalance has been previously described in HIES and may be explained by an intrinsic T cell defect.<sup>28</sup> In our HIES cohort of *STAT3* wt patients, only three of 13 had less than 0.5% IL-17-producing T cells. These three patients may have defects in other proteins involved in *STAT3* signalling or in the differentiation of Th17 cells.

### Proposed set of diagnostic features for *STAT3*-mutant HIES

HIES with mutations in *STAT3* is transmitted as an autosomal dominant trait. Therefore, *STAT3*-mutated HIES patients have a 50% risk of transmitting the disease to each of their offspring.<sup>4,6</sup> Thus, a positive family history contributes to the risk of having HIES. In addition clinical features have been observed to accumulate over time as affected children get older.<sup>1</sup> Hence, any diagnostic algorithm is likely to underdiagnose young patients. However, adding an age feature did not improve our SVM classifier.



## Conclusions

Based on data from our multicenter cohort, we propose the following diagnostic guidelines for *STAT3*-mutant HIES:

*Possible: IgE  $\geq$ 1000 IU/mL plus a weighted score of clinical features  $>$ 30 based on recurrent pneumonia, newborn rash, pathologic bone fractures, characteristic face, and high palate (see Table E4).*

*Probable: Above plus lack of Th17 cells or a family history for definitive HIES.*

*Definitive: Above plus a dominant-negative heterozygous mutation in *STAT3*.*

We caution, however, that none of the scores should be used to keep physicians from pursuing a molecular diagnosis in a particular patient. HIES patients accrue findings over time. Aggressive treatment with antibiotics can forestall infectious complications that would be diagnostic if allowed to occur. Moreover, the unique feature of HIES that abscesses or other infections are 'cold', or not accompanied by a normal intensity of pain or inflammation, has not been captured by any score, but should increase clinical suspicion. HIES scoring will not replace good clinical judgment, but may help the diagnostic process.

The aim of the proposed guidelines is to discern HIES patients carrying mutations in *STAT3* to facilitate time- and resource-saving diagnosis of patients, thereby leading to an early and effective treatment. This screening tool will need to be evaluated and updated as new knowledge is revealed and new mutations causing HIES are identified. We acknowledge that most clinicians will continue to diagnose HIES with its key clinical features such as recurrent pneumonia, lung cysts, typical facies, retention of primary teeth and pathological fractures, When it comes to the question whether or not to evaluate *STAT3*, however, the above scoring should be used.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations Used

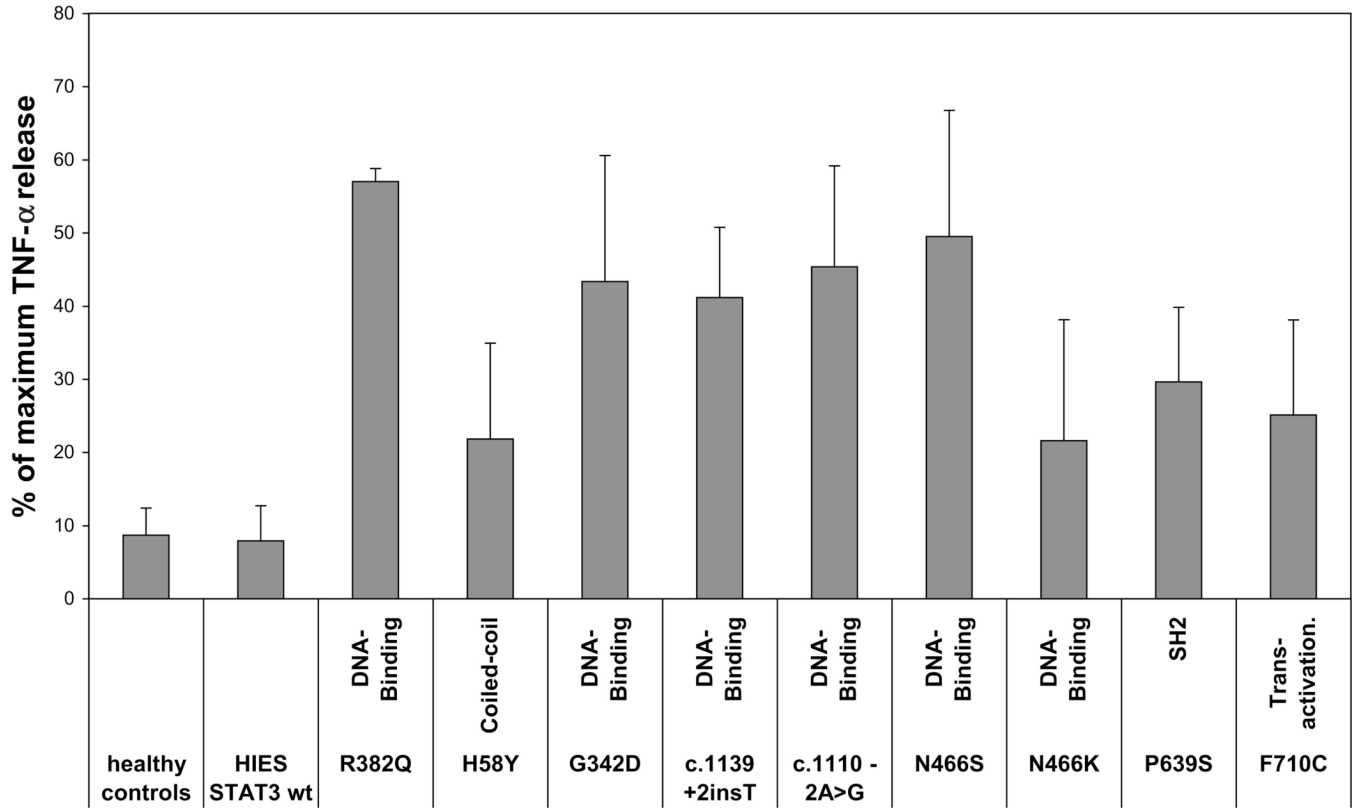
HIES	Hyper-IgE Syndrome
cDNA	complementary DNA
gDNA	genomic DNA
NIH	National Institutes of Health

PBMC	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetate
SEB	Staphylococcus enterotoxin B
SH2	Src homology 2
STAT	Signal transducer and activator of transcription
SVM	Support vector machine
UPN	Unique patient number

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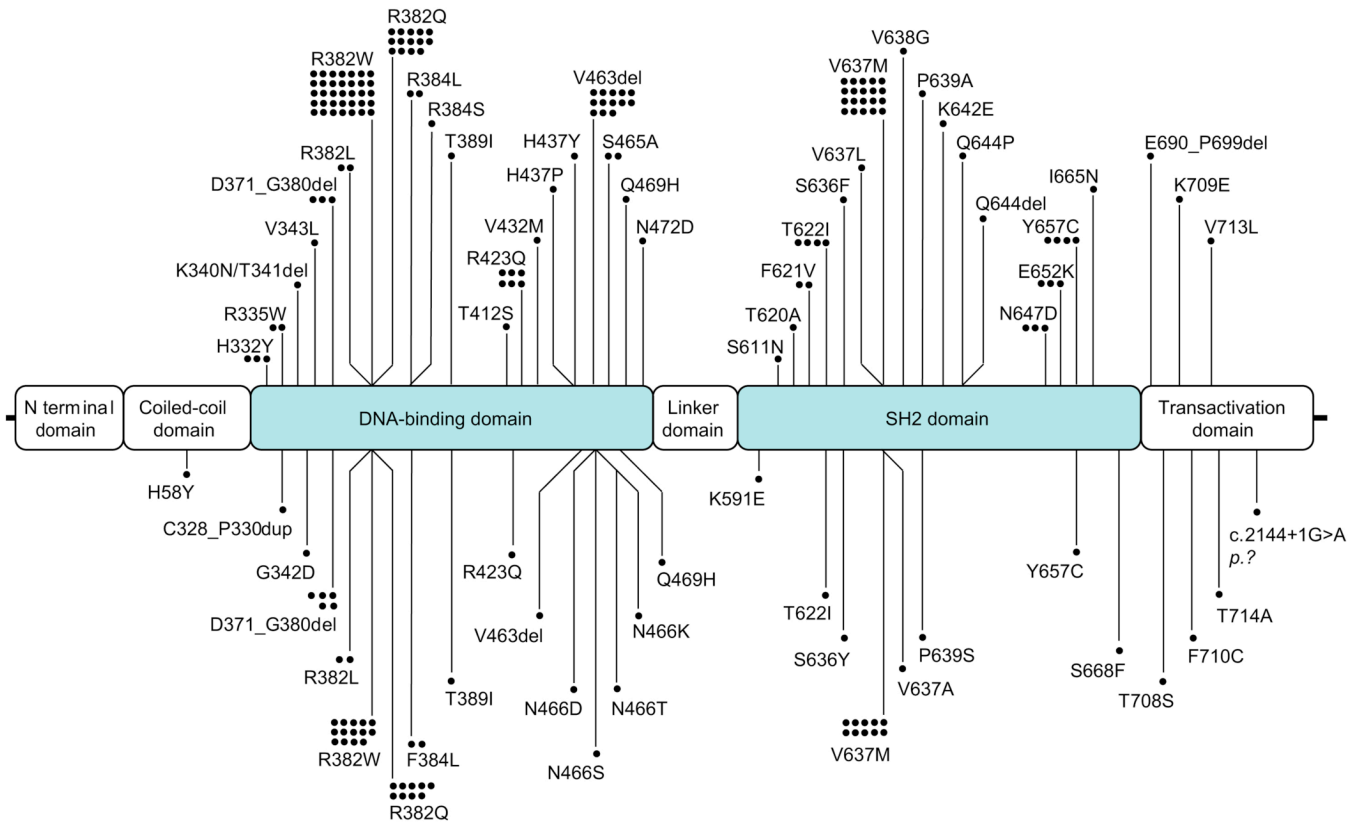
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**Figure 1.** Inhibition of TNF- $\alpha$  release in LPS-activated macrophages by IL-10. Macrophages of nine *STAT3*-mutated patients, eight of whom had novel mutations, one *STAT3* wt patient and eleven healthy controls were pre-treated with IL-10 and then stimulated with LPS. Supernatants were examined for the presence of TNF- $\alpha$ . In order to ensure better comparability of data from different healthy donors, the impact of IL-10 on TNF- $\alpha$  release is shown as percentage of maximum TNF- $\alpha$  release upon LPS stimulation.







**Figure 3.** Schematic structure of STAT3. Every black dot represents one patient carrying the mutation. Patients that carry *STAT3* mutations and that were described in previous publication are shown in the upper part of the figure.<sup>5,6, 13–17</sup> The 64 patients identified in this study who had *STAT3* mutations are shown in the lower part. The patient carrying a splice site mutation after exon 22 is shown as having DNA sequence change as the effect on the protein is not known. *p.?*, unknown effect on protein level

Number and percentage of patients positive for the 17 evaluated features. Features were considered as present if more than one point of the NIH score<sup>4</sup> was achieved. The five cardinal clinical features (recurrent pneumonia, newborn rash, pathologic bone fractures, characteristic face and cathedral palate) are shaded.

Table 1

	all HIES	HIES STAT3 wt	HIES STAT3 mutated			
	%	%	%			
Recurrent pneumonia	85/100	85	24/36	66.7	61/64	95.3
Eczema	90/100	90	32/36	88.9	58/64	90.6
Recurrent skin abscesses	86/100	86	28/36	77.8	58/64	90.6
Characteristic face	82/99	82.8	24/35	68.6	58/64	90.6
Failure to shed deciduous teeth	60/86	68.9	16/31	51.6	44/55	80.0
Lung cyst formation	61/97	62.9	14/34	41.2	47/63	74.6
Eosinophilia	68/94	72.3	27/36	75.0	41/58	70.7
Newborn rash	52/86	60.5	15/29	51.7	37/57	64.9
Other unusual infections	47/94	50	13/34	38.2	34/60	56.7
Increased inter-alar distance	37/83	44.6	10/31	32.3	27/52	51.9
Cathedral palate	41/84	48.8	12/31	38.7	29/53	54.7
Hyperextensibility	37/87	42.5	8/32	25.0	29/55	52.7
Pathologic bone fractures	32/94	34.0	5/35	14.3	27/59	45.8
Recurrent upper respiratory tract infections	41/92	44.6	14/33	42.4	27/59	45.8
Candidiasis	37/91	40.6	12/33	36.4	25/58	43.1
Scoliosis	20/83	24.1	7/33	21.2	13/50	26.0
Midline anomaly	12/86	14.0	5/34	14.7	7/52	13.5

**Table II**

Among the 100 patients studied, 64 patients had 31 distinct mutations. Thirteen of these mutations were reported previously.<sup>5,6, 13–17</sup> The other 18 distinct mutations, which are shaded in this table, have not been previously reported. The cDNA sequence positions reported are from isoform NM\_139276.2. The amino acid positions from the isoform NP\_644805.1.

Number of patients	Protein domain	Site of mutation	DNA sequence change	Predicted amino acid change
1	Coiled-coil	Exon 3	c.172C>T	H58Y
1	DNA-Binding	Exon 10	c.982_990dupTGCATGCC	C328_P330dup
1	DNA-Binding	Exon 10	c.1025G>A	G342D
2	DNA-Binding	Intron 11	c.1110-2A>G	D371_G380del
1	DNA-Binding	Intron 11	c.1110-1G>T	D371_G380del
1	DNA-Binding	Intron 12	c.1139+1G>T	D371_G380del
1	DNA-Binding	Intron 12	c.1139+2insT	D371_G380del
14	DNA-Binding	Exon 13	c.1144C>T	R382W
2	DNA-Binding	Exon 13	c.1145G>T	R382L
9	DNA-Binding	Exon 13	c.1145G>A	R382Q
2	DNA-Binding	Exon 13	c.1150T>C	F384L
1	DNA-Binding	Exon 13	c.1166C>T	T389I
1	DNA-Binding	Exon 14	c.1268G>A	R423Q
1	DNA-Binding	Exon 16	c.1387_1389delGTG	V463del
1	DNA-Binding	Exon 16	c. 1396 A>G	N466D
1	DNA-Binding	Exon 16	c.1397A>G	N466S
1	DNA-Binding	Exon 16	c.1397A>C	N466T
1	DNA-Binding	Exon 16	c.1398C>G	N466K
1	DNA-Binding	Exon 16	c.1407G>T	Q469H
1	SH2	Exon 20	c.1771A>G	K591E
1	SH2	Exon 20	c.1865C>T	T622I
1	SH2	Exon 21	c.1907C>A	S636Y
10	SH2	Exon 21	c.1909G>A	V637M
1	SH2	Exon 21	c.1910T>C	V637A
1	SH2	Exon 21	c.1915T>C	P639S
1	SH2	Exon 21	c.1970A>G	Y657C
1	SH2	Exon 21	c.2003C>T	S668F
1	Transactivation	Exon 22	c.2124C>G	T708S

Number of patients	Protein domain	Site of mutation	DNA sequence change	Predicted amino acid change
1	Transactivation	Exon 22	c.2129T>C	F710C
1	Transactivation	Exon 22	c.2141C>G	T714A
1	Transactivation	Intron 22	c.2144+1G>A	p.?

p.?, unknown effect on protein level

**Table III**

Percentages of IL-17 and IFN- $\gamma$  expressing CD4<sup>+</sup> memory T cells in 30 analyzed patients

UPN	Gender	Age of scoring	Origin	DNA sequence change	Predicted amino acid change	Domain	NIH score*	STAT3 deficiency score <sup>§</sup>	% of CD45RO <sup>+</sup> IL17 <sup>+</sup> IFN- $\gamma$ <sup>-</sup> cells	% of CD45RO <sup>+</sup> IL17 <sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells	Candidiasis
110	F	28	EU	c.1025G>A	G342D	DNA-Binding	79	63.33	0.27	11.44	yes
114	M	22	EU	c.1110-2A>G	D371_G380del	DNA-Binding	70	50.00	0.32	5.57	yes
086	M	13	EU	c.1110-1G>T	D371_G380del	DNA-Binding	83	76.67	0.13	5.80	yes
017	F	25	EU	c.1139+1G>T	D371_G380del	DNA-Binding	65	41.66	0.33	1.43	yes
108	M	14	EU	c.1119+2insT	D371_G380del	DNA-Binding	54	31.67	0.22	3.51	yes
015	M	29	EU	c.1144C>T	R382W	DNA-Binding	76	63.33	0.21	15.76	yes
019	M	15	EU	c.1144C>T	R382W	DNA-Binding	63	48.33	0.71	5.88	yes
126	F	11	EU	c.1144C>T	R382W	DNA-Binding	58	40.00	0.12	7.17	yes
128	F	3	EU	c.1144C>T	R382W	DNA-Binding	29	38.33	0.53	1.73	yes
060	M	29	ME	c.1150T>C	F384L	DNA-Binding	71	55.00	0.68	1.33	yes
118	F	32	EU	c.1268G>A	R423Q	DNA-Binding	59	40.00	0.19	5.43	no
071	M	24	EU	c.1907C>A	S636Y	SH2	71	40.00	0.31	2.36	yes
016	F	39	EU	c.1909G>A	V637M	SH2	68	55.00	0.18	8.90	yes
115	M	19	EU	c.1909G>A	V637M	SH2	56	68.33	0.72	5.72	nd
125	M	16	EU	c.1909G>A	V637M	SH2	58	45.00	0.22	7.79	no
018	M	23	EU	c.2129T>C	F710C	Transactivation	58	40.00	0.46	11.41	yes
116	M	8	EU	c.2141C>G	T714A	Transactivation	63	50.00	0.40	5.59	yes
001	M	19	EU	no mutation	no mutation		72	63.33	0.25	2.45	yes
004	M	17	EU	no mutation	no mutation		60	35.00	0.79	10.46	yes
009	M	30	EU	no mutation	no mutation		49	25.00	0.69	1.49	yes
010	M	59	EU	no mutation	no mutation		29	25.00	0.18	0.65	no
014	F	34	EU	no mutation	no mutation		47	21.67	0.28	1.80	no
020	F	9	EU	no mutation	no mutation		32	21.67	1.00	3.28	no
022	F	18	EU	no mutation	no mutation		43	25.00	0.54	2.53	yes
054	M	21	EU	no mutation	no mutation		29	20.00	0.83	1.01	yes
069	M	25	EU	no mutation	no mutation		61	48.33	1.03	4.06	yes



UPN	Gender	Age of scoring	Origin	DNA sequence change	Predicted amino acid change	Domain	NIH score*	STAT3 deficiency score <sup>§</sup>	% of CD45RO <sup>+</sup> IL17 <sup>+</sup> IFN- $\gamma$ <sup>-</sup> cells	% of CD45RO <sup>+</sup> IL17 <sup>-</sup> IFN- $\gamma$ <sup>+</sup> cells	Candidiasis
<b>097</b>	M	5	EU	no mutation	no mutation		39	20.00	0.81	3.56	yes
<b>113</b>	F	5	EU	no mutation	no mutation		39	20.00	0.73	0.67	no
<b>124</b>	M	1	EU	no mutation	no mutation		22	5.00	1.21	0.47	nd
<b>127</b>	F	7	EU	no mutation	no mutation		49	26.67	0.77	3.80	yes

UPN, unique patient number

F, Female; M, Male

EU, Europe; ME, Middle East

\* scoring system described in Grimmacher et al.<sup>4</sup>

<sup>§</sup> weighted score based on the five cardinal features (recurrent pneumonia, newborn rash, pathologic bone fractures, a characteristic face, and a cathedral palate) shown in Table E4 in the Online Repository. A total number of scaled points greater than 30.0 predicts a *STAT3* mutation.

Patients with less than 0.5% CD45RO<sup>+</sup> IL17<sup>+</sup> IFN- $\gamma$ <sup>-</sup> or 5% CD45RO<sup>+</sup> IL17<sup>-</sup> IFN- $\gamma$ <sup>+</sup> cells are shaded.