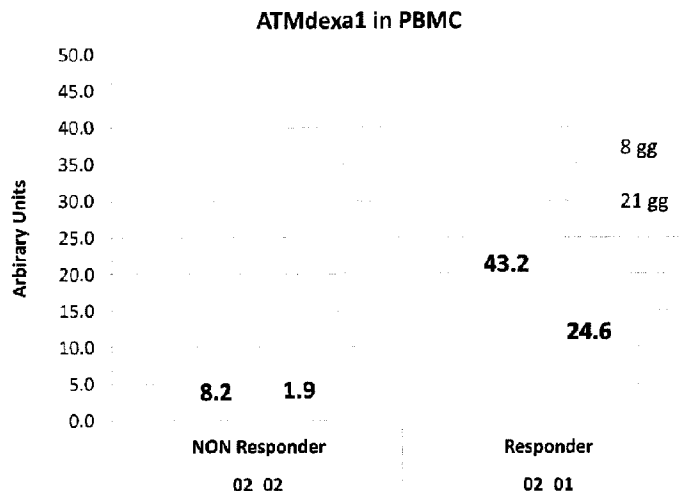




(86) Date de dépôt PCT/PCT Filing Date: 2016/01/19
(87) Date publication PCT/PCT Publication Date: 2016/07/28
(45) Date de délivrance/Issue Date: 2022/06/21
(85) Entrée phase nationale/National Entry: 2017/07/18
(86) N° demande PCT/PCT Application No.: IB 2016/050238
(87) N° publication PCT/PCT Publication No.: 2016/116850
(30) Priorité/Priority: 2015/01/19 (IT RM2015A000022)

(51) Cl.Int./Int.Cl. *C12Q 1/68* (2018.01),
A61K 31/573 (2006.01), *A61P 25/28* (2006.01),
C12Q 1/6809 (2018.01), *C12Q 1/6876* (2018.01)
(72) Inventeurs/Inventors:
MAGNANI, MAURO, IT;
BIAGIOTTI, SARA, IT;
MENOTTA, MICHELE, IT
(73) Propriétaire/Owner:
ERYDEL S.P.A., IT
(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(54) Titre : PROCEDE D'EVALUATION DE LA REPONSE DE PATIENTS AFFECTES PAR L'ATAXIE TELANGIECTASIE
AU TRAITEMENT PAR LES GLUCOCORTICOIDES
(54) Title: METHOD OF EVALUATING THE RESPONSE OF ATAXIA TELANGIECTASIA PATIENTS TO
GLUCOCORTICOIDS TREATMENT



(57) **Abrégé/Abstract:**

The present invention relates to a novel procedure for evaluating the response of patients affected by Ataxia Talangiectasia (A-T) to glucocorticoids treatment. In particular, the procedure provides a step of qualitative and/or quantitative identification in the blood of said patients of the expression of a mRNA variant of the ATM (Ataxia-Talangiectasia-Mutated) gene produced by non-canonical splicing induced by glucocorticoid (GC). In fact, it was demonstrated that mRNA variant expression is present in the blood of patients who responded positively to treatment with GC.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(10) International Publication Number
WO 2016/116850 A1

(43) International Publication Date
28 July 2016 (28.07.2016)

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/IB2016/050238

(22) International Filing Date:
19 January 2016 (19.01.2016)

(25) Filing Language: Italian

(26) Publication Language: English

(30) Priority Data:
RM2015A000022 19 January 2015 (19.01.2015) IT

(71) Applicant: ERYDEL S.P.A. [IT/IT]; Via Sasso 36, 61029 Urbino PU (IT).

(72) Inventors: MAGNANI, Mauro; Via Ca Adelia 6, 61029 Urbino PU (IT). BIAGIOTTI, Sara; Via G. Donizetti 24A, 61010 Tavullia PU (IT). MENOTTA, Michele; Via A. Doria 56, 61032 Fano PU (IT).

(74) Agents: GERMINARIO, Claudio et al.; c/o SOCIETA' ITALIANA BREVETTI S.p.A., Piazza di Pietra, 39, 00186 Rome (IT).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: METHOD OF EVALUATING THE RESPONSE OF ATAXIA TELANGIECTASIA PATIENTS TO GLUCOCORTIC-
OIDS TREATMENT

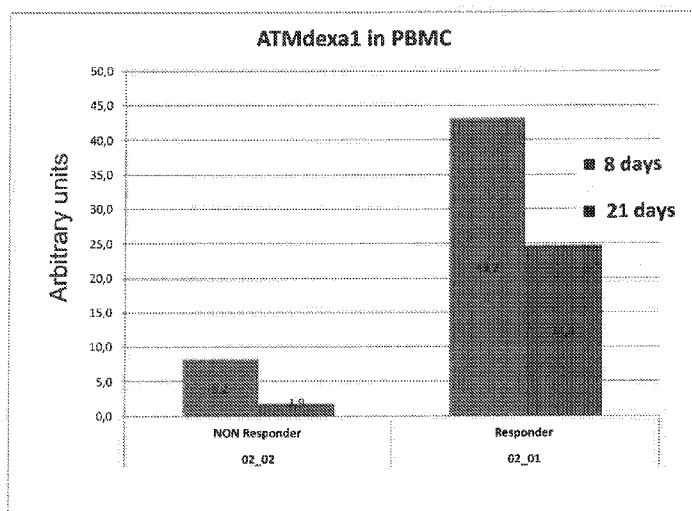


FIGURE 2

(57) Abstract: The present invention relates to a novel procedure for evaluating the response of patients affected by Ataxia Telangiectasia (A-T) to glucocorticoids treatment. In particular, the procedure provides a step of qualitative and/or quantitative identification in the blood of said patients of the expression of a mRNA variant of the ATM (Ataxia-Telangiectasia-Mutated) gene produced by non-canonical splicing induced by glucocorticoid (GC). In fact, it was demonstrated that mRNA variant expression is present in the blood of patients who responded positively to treatment with GC.

METHOD OF EVALUATING THE RESPONSE OF ATAXIA TELANGIECTASIA PATIENTS TO
GLUCOCORTICIDS TREATMENT

5 FIELD OF THE INVENTION

The present invention relates to a novel procedure for evaluating the response of patients affected by Ataxia Talangiectasia (A-T) to glucocorticoids treatment. In particular, the procedure provides a step of qualitative and/or quantitative identification in the blood of said patients of the expression of a mRNA variant
10 of the ATM (Ataxia Talangiectasia-Mutated) gene produced by non-canonical splicing induced by glucocorticoid (GC). In fact, it was demonstrated that mRNA variant expression is present in the blood of patients who responded positively to treatment with GC.

15 STATE OF THE PRIOR ART

In the last years, various clinical trials provided evidences that short-term treatment with glucocorticoids (GC) is able to improve neurological symptoms in A-T patients, and even the state of cerebellar atrophy in some subjects [1-4]. Unfortunately, such an improvement is merely transitory and disappears shortly
20 after discontinuation of oral treatment with GC; discontinuation is necessary, as the hazards of a long-term therapy with steroids would risk to surpass its benefits [5]. On the other hand, the administration of very low GC doses by erythrocytes could reduce steroids toxicity without compromising their effectiveness [6-10]. Therefore, in 2010 a phase II Clinical study was set up
25 which envisaged the long-term treatment of A-T patients by dexamethasone encapsulated within autologous erythrocytes [11]. The proposed therapy brought about a significant improvement in neurological symptoms, concomitantly avoiding the onset of known side effects typical of GC.

Recently, *in vitro* experiments, conducted on stabilized lymphoblastoid cell lines from lymphocytes of A-T patients, allowed the Inventors to demonstrate that the action of synthetic GC dexamethasone (dexa) action may be exerted through synthesis of a new messenger RNA (mRNA) molecule generated by a non-
5 canonical splicing event in the pre-mRNA precursor of the ATM gene (Ataxia Telangiectasia Mutated gene).

In silico studies suggested that the resulting transcript, referred to by the Inventors as ATMdexa1, instead of being junk RNA, could be translated by
10 cellular mechanisms into a new "shortened" form of the ATM protein, skipping all mutations, present in the gene of AT patients, upstream of the sequence encoding the kinase and functional enzyme domain. This protein variant, named mini-ATM, was effectively identified in the lymphoblastoid cell lines and demonstrated to be potentially active. These findings led the Inventors to
15 hypothesize that dexa treatment may restore, at least partly, the defective ATM protein in Ataxia telangiectasia by means of this novel molecular mechanism that, surprisingly, could allow to "skip" most of the mutations so far described for the *ATM* gene.

20 SUMMARY OF THE INVENTION

The present invention is based on the discovery that a new transcript of mRNA variant of the ATM gene (Ataxia-Talangiectasia-Mutated gene) produced by non-canonical splicing induced by glucocorticoids (GC) treatment is identifiable in blood samples obtained from A-T patients subjected to treatment with GC, and
25 that its presence correlates with positive response to therapeutic treatment.

Hence, object of the present patent application is a novel procedure for quantitatively or qualitatively analyzing the mRNA variant of the ATM gene as biomarker of response to glucocorticoids treatment, such as dexamethasone, in
30 the blood of patients. In fact, it was demonstrated by the present inventors that

the expression of said mRNA variant is present in the blood of patients that responded positively to GC treatment, above all when the medicament is encapsulated within erythrocytes, but is absent in healthy controls and in patients not receiving the drug.

5 Hence, objects of the present application are:

A method for evaluating the response of patients affected by Ataxia Talangiectasia to glucocorticoids treatment, comprising a step of qualitative and/or quantitative identification in the blood of said patients of the expression of a mRNA variant of the gene ATM (Ataxia-Talangiectasia-Mutated gene),
10 wherein said mRNA variant is produced by non-canonical splicing induced by glucocorticoid and contains the Phosphatidyl Inositol 3 Kinase domain, and wherein detectable expression values of said mRNA variant indicate positive response to treatment.

15 A method for treating patients affected by Ataxia Talangiectasia, comprising

- a step of administering an amount of glucocorticoide, preferably dexamethasone, in said patient;

- a step of qualitative and/or quantitative identification in the blood of said patients of the expression of a mRNA variant of the gene ATM (Ataxia-Talangiectasia- Mutated gene) wherein said mRNA variant is produced by non-canonical splicing induced by glucocorticoid and contains the Phosphatidyl Inositol 3 Kinase domain, and wherein detectable expression values of said mRNA variant indicate positive response to treatment;

- a step of adjusting said therapy depending on the response.

25 In one embodiment of the invention, the glucocorticoid is selected from: prednisolone, dexamethasone, betamethasone, deflazacort, or pharmaceutically acceptable salts thereof.

In another embodiment of the invention, the mRNA variant of the ATM gene is ATMdexa1 mRNA.

In a further embodiment, the identification of the expression of the mRNA variant is quantitative and is normalized against the expression of a
5 housekeeping gene.

In a further embodiment of the invention, the identification of the mRNA variant is performed by any technique of amplification using suitable 5'-3' forward and 5'-3' reverse primer pairs, optionally associated with probes.

In an alternative embodiment of the invention, the glucocorticoide is
10 encapsulated within erythrocytes.

The advantages afforded by the present invention are immediately evident to a person skilled in the art, when considering that the treatment with corticosteroids is often accompanied by adverse effects, even serious ones, above all when the treatment is prolonged over time and/or conducted on a
15 pediatric population. Apparently, the prognostic tool afforded by the present invention avoids the prolonging of treatments in subjects not responding (non-responders) or not sufficiently responding to therapy, and therefore of useless and harmful treatments. Moreover, by measuring ATMdexa1 expression levels it is possible to establish the optimal frequency of glucocorticoids administration
20 in a given patient, reducing the risk of overdosage and of adverse effects due to the treatment, yet preserving the therapeutic effects thereof.

The same method could be used to develop new drugs, even different from glucocorticoids, however able to induce ATMdexa1 expression and therefore bring benefits to the treated patient.

25

DESCRIPTION OF THE FIGURES

Figure 1: Qualitative and quantitative identification of ATMdexa1 transcript in blood samples derived from A-T patients treated with ERYDEX. ATMdexa1 expression levels were detected by RT-PCR method with SYBR™green and
30 compared to the expression levels of untreated A-T patients and untreated WT

healthy volunteers (panel **a**). In a second analysis, the A-T patients treated with ERYDEX were subdivided into two subgroups (responders and non-responders) based on responsiveness to treatment measured with the ICARS scale (Trouillas P, et al. "International Cooperative Ataxia Rating Scale for pharmacological assessment of the cerebellar syndrome. - The Ataxia Neuropharmacology Committee of the World Federation of Neurology". *J Neurol Sci.* 1997 Feb 12;145(2):205-11.)

and the related expression levels compared between the two treated subgroups and with the healthy and untreated controls (panel **b**). As non-responders, in the figure, are considered the patients who, following the treatment, had demonstrated an ICARS value decrease lower than or equal to 10 points, after six months of therapy.

Figure 2: ATMdexa1 quantification at different times from treatment. For a responder patient and a non-responder patient, expression levels were evaluated at +8 and +21 days from ERYDEX administration.

Figure 3: Exemplary standard curve of ATMdexa1 amplification by RT-PCR method and SYBRgreen. Amplification (panel **a**), dissociation (panel **b**) and linearity (panel **c**) curves were obtained by amplifying serial dilutions of a suitable recombinant plasmid for ATMdexa1.

Figure 4: ATMdexa1 amplification (panel **a**) and dissociation (panel **b**) curves in samples of A-T patients treated with ERYDEX.

Figure 5: Analysis of the linear relationship existing between miniATM expression levels and ICARS variation depending on the administered amount of dexamethasone (Dexa) (Figure **a**). Confirmation of data related to responsiveness to treatment with ERYDEX and miniATM expression levels between responders and non-responders, and respective ICARS values (Figure **b**).

Figure 6: Assessment of expression of FKBP5 and DUSP1 genes, whose transcription is induced by dexamethasone administration, in responder and non-responder patients treated with Erydex.

DETAILED DESCRIPTION OF THE INVENTION

The ATM gene (Ataxia Telangiectasia Mutated gene) was described by Savitsky K, et al. In Science. 1995 Jun 23;268(5218):1749-53. The gene, which
5 codifies for a protein kinase of the PI 3-kinase family, is transcribed into 27 different mRNAs and into 20 known variant forms of mRNA due to alternative splicing.

New transcripts of mRNA variants, relevant for the purposes of the present invention, are detectable in the blood of patients affected by Ataxia
10 Telangiectasia subjected to glucocorticoids treatment, and linked to the therapeutic effect on AT of the same glucocorticoids.

Said transcripts are the result of an alternative non-canonical splicing, induced by glucocorticoids, which, by markedly limiting their length with respect to physiological transcripts, removes the mutation sites of the ATM gene, however
15 at least partly preserving its region accountable for the protein kinase enzymatic activity, containing the Phosphatidyl Inositol 3 Kinase domain.

In particular, a previous experimental work, carried out using as exemplary glucocorticoid Dexamethasone sodium phosphate, enabled to identify *in vitro*, on cell lines of A-T patients, a transcript of about 1582 bp denominated
20 ATMdexa1 (see M. Menotta et al J. Biological Chemistry Vol 287, N. 49 of November 30, 2012).

It has been demonstrated that the administration of dexamethasone derivatives, such as salts different from sodium phosphate, or of glucocorticoids of the same family of dexamethasone, equally known for exerting an improving therapeutic
25 effect on the clinical picture of Ataxia Telangiectasia, act through the same mechanism, inducing the same transcript of mRNA variant or functionally equivalent transcripts.

It has now been observed by the present Inventors that ATMdexa1 synthesis is
30 directly correlated to the treatment with the drug, since neither A-T subjects, nor

WT subjects, who did not receive dexamethasone treatment, demonstrated detectable levels of transcript.

For the purposes of the present invention, by the wording “*detectable levels of transcript*” or “*detectable expression values*” are meant values that, regardless of detection methodology and operating conditions (e.g., number of cycles or temperature of a PCR) be greater than the average values of the controls (i.e., A-T patients not treated with GC or healthy individuals) of at least 3 times the standard deviation of the reference sample.

Moreover, *ATMdexa1* expression is directly and proportionally correlated to clinical effectiveness, it being greater in those patients with a greater decrease of overall symptoms of the disease (i.e., decrease of the ICARS score with entailed Δ ICARS increase) and associated with a greater improvement of neurological symptoms (responder patients) compared to patients for which no significant improvements of disease symptoms were observed (non-responder patients). Non-responders are considered the patients who, following the treatment, have demonstrated a decrease of the ICARS value lower than or equal to 10 points, after six months of therapy.

Moreover, it was demonstrated that the expression of genes, such as FKBP5 and DUSP1, known to be more expressed in dexamethasone treatment, does not vary in the two patient groups (responders and non-responders) therefore highlighting the specificity of miniATM as specific biomarker of therapy effectiveness.

Lastly, *ATMdexa1* expression was demonstrated greater in the days immediately after medicament infusion and slowly decreases over time, along with the disappearance of the circulating drug, confirming a relationship between Dexamethasone administration, clinical response, and *ATMdexa1* expression.

The treatments and patients susceptible of being monitored with the method of the present invention are treatments of AT with administration of glucocorticoids known to positively affect the clinical picture of the disease. Glucocorticoids are selected from the family comprising prednisolone, dexamethasone, 5 betamethasone, deflazacort, derivatives thereof and salt forms thereof, such as phosphate diacid or sodium phosphate, for instance dexamethasone phosphate, dexamethasone sodium phosphate, betamethasone phosphate or sodium phosphate, prednisolone phosphate or sodium phosphate, deflazacort phosphate or sodium phosphate. Evidently, the forms salified with sodium can 10 be replaced by the corresponding and usual salts of group IA or IIA metals without altering the essence of the invention.

In a specific form of treatment monitorable thorough the method of the invention, the medicament is administered in a form encapsulated within erythrocytes. The encapsulation process is described in Appn. WO2014/181309 15 (PCT/IB2014/061338) entitled: "*Process for the Preparation of Erythrocytes Loaded with One or More Substances of Pharmaceutical Interest and so Obtained Erythrocytes*".

The experimental work described in the present application was carried out by using dexamethasone sodium phosphate encapsulated within erythrocytes 20 according to the method described in WO2014/181309.

Various methods, all based on polymerase chain reaction, were contrived and set up for identification and quantification of the expression of the mRNA variant, e.g. of ATMdexa1, in the collected blood sample. All methodologies 25 introduced herein allow relative and/or absolute quantification of messenger RNA in the sample of interest.

By way of example, the amplification is carried out on blood samples or on peripheral mononuclear cells (PBMC), by techniques selected from: PCR, RT- 30 PCR, RT-PCR with an intercalating agent, Taq polymerase-PCR, Molecular

Beacon probe method PCR, FRET-probe hybridization, Scorpion probe PCR. The protocols for each technology are described in the experimental section of the present application and are object of the invention.

As quantification method, it was preferably used that of the standard curve set up from a molecule of recombinant plasmid for the sequence of the mRNA transcript (e.g., ATMdexa1) added in known and increasing amounts in DNA samples used as standard to evaluate amplification effectiveness and method linearity. The same serial dilutions of the plasmid/mRNA can be used as PCR positive control and as gold standard for absolute quantification of the target molecule. Alternatively, the assay provides a pair of primers for amplifying, concomitantly with the target, the mRNA of HPRT1, selected as housekeeping gene in the sample of interest. Besides HPRT1, other reference genes were tested (e.g., GAPDH, β 2M, etc.) and might be used for the relative quantification of ATMdexa1 thanks to the $\Delta\Delta C_t$ or Pfaffel methods.

In accordance with the various technologies, suitable *forward* and *reverse* primers were designed and implemented by the present Inventors. The nucleotide sequences of said primers are listed in the sequence listing and in Table 1 below.

According to the technology employed, suitable probes for amplificate detection are also used. The sequences of some of these probes are reported in the sequence listing and in Table 1.

Table 1

Method	Forward Primer 5'-----3'	Reverse Primer 5'-----3'	Probe 5'-----3'	Reporter/ Fluorophore/ Donor	Quence r/ Fluorop hore	Quantifi cation
SYBRgreen n qRT-PCR	ATCTAGATC GGCATTCA GATTCCA	GCAGACCAG CCAATTACT AAAC	none	SYBRgreen	none	Direct

5' Nuclease assay	CGCCTGATT CGAGATCC TGAA	GTGCCTCAA CACTTCTGA CCAT	AAACATCTAGAT CGGCATTGAGAT TCCAA	Cy3	BHQ2	
5' Nuclease assay	CGCCTGATT CGAGATCC TGAA	GTGCCTCAA CACTTCTGA CCAT	TCTAGATCGGCA TTCAGATTCC	Cy3	BHQ2-Plus	
Molecular Beacons	AGAATGTCT GAGAATAG CA	AACTTAGAT GCCACTCAG	GATGGTCAGAAG TGTTGAGG	6-FAM	BHQ1	Molecular Beacon
Hybridization/ FRET Probes	ATCCTGAAA CAATTAAC AT	ACTTTGTGA TGCTTATATT AT	TTTACAGAAATA TATTCAGAAAAGA AACAGA/ TGAGAATAGCAA AACCAAATGTAT C	FAM	LC red 640	

Lastly, examples of primers and probes for amplification of known housekeeping genes, used as positive control in the amplification processes according to the invention, are reported in Table 2.

5

Table 2

Method	Forward Primer 5'-----3'	Reverse Primer 5'-----3'	Probe 5'-----3'	Reporter/ Fluorophore/ Donor	Quencher/ Fluorophore	Quantification
SYBRgreen qRT-PCR	TATGCTGAG GATTTGGAA AGGGT	CCATCACAT TGAGCCCT CT	none	SYBRgreen	none	Direct
5' Nuclease assay	GGAAAGGG TGTTTATTC CTCATGGA	GGCCTCCCA TCTCCTTCAT C	TATGGACAGGACT GAACGTCTTGC	JOE	BHQ1	
5' Nuclease assay	GGATTTGG AAAGGGTG TTTATTCC	GGCCTCCCA TCTCCTTCAT C	TGGAATAATTATG GACAGGACTGA	JOE	BHQ1-Plus	
5' Nuclease	TATGCTGAG GATTTGGAA	CCATCACAT TGAGCCCT	TATGGACAGGACT GAACGTCTTGC	JOE	BHQ1	

assay	AGGGT	CT				
Molecular Beacons	TCGTGATTA GTGATGAT GA	TTATGGACA GGACTGAAC	TACCTAATCATTAT GCTGAGGATT	HEX	BHQ1	
Hybridization/FRET Probes	GGCTATAA ATTCTTTGC T	GGACATAAA AGTAATTGG T	AGATCCATTCCTA TGA CTGTAGATT/ CAGACTGAAGAGC TATTGTAATGAC	FAM	LC red 640	

Experimental section

The following section reports, by way of example, an experimental work performed under the IEDAT clinical study, using dexamethasone as representative sample of glucocorticoid medicaments.

In vivo experimentation

Following approval of the ethical committees in charge, and with approval by the patients or their legal guardians if minors, blood samples of 10 out of 22 patients enrolled in the IEDAT clinical study were obtained. Blood collection was carried out at the end of the treatment, precisely at Day 21 from the sixth and latter infusion of intra-erythrocytic dexamethasone (ERYDEX®) provided by the clinical study. The blood sample was also collected in specially provided tubes (vacutainers) containing a stabilizing solution adapted to preserve RNA for the subsequent extraction. In short, the extracted RNA was back transcribed into the corresponding cDNA and subjected to analyses aimed at measuring ATMdexa1 expression. For this purpose, a Real Time PCR assay was set up for qualitative and quantitative analysis, able to specifically identify the new mRNA molecule first described in lymphoblastoid cells. Thanks to said assay, ATMdexa1 was identified in all 10 available samples. The same investigations were carried out on blood samples from A-T patients not treated with Dexamethasone drug and volunteer healthy (WT) subjects, used as control. In said samples, ATMdexa1 was never detected (**Figure 1a**). Reported results

suggest that the induction of ATMdexa1 isoform seems to be strictly dependent on the treatment with the drug.

ERYDEX efficacy on neurological symptoms of A-T patients, evaluated under the study by ICARS scale, revealed that a greater improvement was associated to a greater encapsulation and, therefore, to a greater administration of drug [11]. For this reason, patients were clustered in responders (or “loaders”) and non-responders (or “non-loaders”). To correlate dexa efficacy with ATMdexa1 induction, a subanalysis was performed by subdividing the samples from the patients into two groups. As shown in **Figure 1b**, responder A-T patients (samples) exhibit greater ATMdexa1 expression compared to those from non-responder patients, suggesting that indeed ATMdexa1 expression seems to be directly correlated to, or at least contribute to, dexa efficacy. Lastly, blood samples were analyzed, from a single patient of both groups, collected at different times from ERYDEX infusion, specifically at +8 and +21 days from administration. In this case as well, responder patients demonstrated expression levels greater than non-responders at both times. However, it was surprisingly observed (by comparing intra-patient levels in both cases) that the ATMdexa1 transcript is expressed at greater levels at the +8 day time than at the +21 day time (**Figure 2**). This is consistent with the gradual waning of dexa effect over time from the treatment, a fact stressing the need to resort to repeated administrations in order to keep the drug effectiveness active.

In a subsequent experiment, in which patients were enrolled similarly to what described above, a directly proportional relationship was observed between miniATM expression and ICARS variation depending on the amount of drug (dexamethasone) administered (Figure 5 a). In particular, the correlation between miniATM expression and amount of drug (dexamethasone) administered is of linear type. Moreover, it was confirmed that an increase of miniATM expression correlates with a greater improvement of neurological symptoms, i.e. decrease of ICARS scores and entailed Δ ICARS increase. (figure 5 b).

Lastly, an evaluation of FKBP5 and DUSP1 genes expression in the same samples used for miniATM determination highlighted that, while glucocorticoids treatment always increases the expression of such genes, the expression level does not change significantly in the two groups of patients (responders and non-responders). This observation highlights the specificity of miniATM expression as specific biomarker of therapy efficacy.

To sum up, the new ATMdexa1 transcript recently identified in *ATM*^{-/-} lymphoblastoid cell lines, was first identified *in vivo*, in particular, in PMBCs of A-T patients treated with ERYDEX. ATMdexa1 synthesis is directly correlated to treatment with the drug, as neither A-T subjects, nor WT subjects who did not receive dexamethasone demonstrated detectable levels of transcript. Moreover, *ATMdexa1* expression is directly correlated with clinical efficacy, it being greater in those patients with greater decrease of ICARS score and associated with a greater improvement of neurological symptoms. Lastly, ATMdexa1 expression proved greater in the days immediately subsequent to ERYDEX infusion and slowly decreases over time, in parallel with the disappearance of circulating drug, confirming an association between Dexamethasone administration, clinical response and ATMdexa1 expression.

20

Experimental protocol for ATMdexa1 mRNA identification and quantification in PMBCs of A-T patients treated with dexamethasone

1) Samples collection:

3ml of blood were collected from A-T patients and healthy volunteers directly in vacutainer Tempus Blood RNA tubes (Applied Biosystems), containing a lysing solution and a stabilizing reagent that assures RNA preservation until subsequent extraction. Immediately after withdrawal, samples were frozen and could be kept at -20°C for several months. Shipment from hospital centers to

the Inventors' laboratory was carried out at the same temperature to avoid sample defrosting and deterioration.

2) Total RNA extraction and complementary DNA synthesis:

Blood samples were processed for total RNA extraction by Tempus spin RNA
5 isolation kit (Applied Biosystems) as reported in the manufacturer's instructions. A cleanup process was made to follow a previous purification by QIAGEN RNA extraction kit for a complete elimination of contaminating DNA. The average yield was about 8.5 micrograms of pure total RNA for each sample, consisting at the start of 3ml of whole blood. Then, 500 ng RNA from each sample were
10 employed for retro-transcription reaction into the corresponding complementary DNA (cDNA), catalyzed by the enzyme SMARTScribe Reverse Transcriptase (Clontech) in the presence of oligodT or Random hexamers or both, to prime the reaction. cDNAs synthesis was conducted as reported in the protocol illustrated by the manufacturer.

15 Exogenous, not conserved RNA molecule, obtained from a species other than Homo sapiens (e.g., bacterial or plant mRNA), was added prior to RNA extraction and cDNA synthesis procedures, as internal control, so as to be able to verify extraction, synthesis and subsequent amplification efficiencies. More preferably, the molecule used as standard may be added directly into the
20 stabilizing solution contained into the vacutainer.

3) PCR assay for ATMdexa1 quantification in the samples:

Several methods, all based on polymerase chain reaction (PCR), have been contrived and set up for ATMdexa1 identification and quantification in the
25 collected blood sample; some of them are described in detail hereinafter and therefore claimed as invention. All the methodologies introduced here permit the relative and/or absolute quantification of the ATMdexa1 messenger RNA in the sample of interest.

a) The first method claimed envisages the use of an intercalating agent, like, e.g., SYBRgreen or any other fluorophore having intercalating action, into the double strand (ds) of the DNA. Such a technology is based on the specific amplification of a cDNA family by quantitative real-time PCR in the presence of the intercalating agent that intercalates into the double strand of the increasing DNA to monitor amplicon synthesis. Specifically, for their experiments the Inventor used SYBR Premix Ex Taq (Tli RNaseH Plus) by Takara, containing the enzyme Taq polymerase and SYBRgreen in a suitable buffer system. An additional amount of $MgCl_2$ was added to the reaction mixture to reach the optimal final concentration of 3.5 mM, as well as a pair of specific primers at a final concentration of 300 nM. The oligonucleotide pair was designed *in silico* to recognize selectively the DNA complementary to the ATMdexa1 transcript (ATMdexa1 cDNA) to the detriment of native mRNA (Tables 1 and 2). In the example proposed herein, raw fluorescence, measured in real time in the samples under amplification, will be strictly dependent on the ATMdexa1 RNA quantity present in the sample of origin, and will employ a number of amplification cycles inversely proportional to the initial RNA quantity, to reach the threshold level of fluorescence. The thermal profile optimized to obtain a specific and linear amplification of ATMdexa1 was set in 40 cycles of denaturation (for 10 sec at 94°C), annealing (for 20 sec at 65°C) and extension at 72°C for 36 seconds. As method for quantification, it was used that of a standard curve assembled from a molecule of plasmid recombinant for the ATMdexa1 sequence added in known and increasing quantities in DNA samples used as standard to assess amplification efficiency and linearity of the method. The same serial dilutions of ATMdexa1 plasmid can be used as PCR positive control and as gold standard for absolute quantification of the target molecule. Alternatively, the assay envisages a pair of primers to amplify in the sample of interest, concomitantly with the target, the HPRT1 mRNA selected as housekeeping gene. Besides HPRT1, other reference genes were tested (e.g.,

GAPDH, β 2M, etc.) and might be used for the relative quantification of ATMdexa1 thanks to the $\Delta\Delta C_t$ or Pfaffel methods.

The experimental results, given in the description of the invention, were obtained by means of the assay described in this section. Method validation
5 envisaged the setting up of a standard curve as described above. The related amplification (Figure 3, panel A), dissociation (Figure 3, panel B) and linearity (Figure 3, panel C) curves are reported in Figure 3, which demonstrates a specific, linear and univocal amplification of ATMdexa1. The extrapolated linearity line has a slope of -3.30 (corresponding to an amplification efficiency of
10 100%) and an R^2 of 0.99.

Samples of interest, from A-T patients subjected to dexamethasone treatment, were amplified under the same conditions and compared with those of untreated A-T patients and healthy volunteers. Specific amplification was obtained in all investigation samples, as shown in Figure 4, whereas no amplification was
15 detected in the control samples. Threshold cycles (C_t) derived for positive samples were used for calculation of the relative amounts of ATMdexa1 mRNA subtracting the related threshold C_t of the housekeeping HPRT1.

b) The second method exemplified is based on 5'-Nuclease Assay,
20 exploiting the 5'-exonuclease activity of Taq polymerase enzyme. Besides the pair of specific primers, a likewise specific DNA probe is used, which anneals on the newly synthesized amplicon and is hydrolyzed by polymerase during chain extension. The probe bears, bound to the 3'- and 5'-ends, respectively a quencher (BHQ or BHQPlus quencher) and a reporter fluorophore, where the
25 former inhibits emission of the latter. Hydrolysis of the probe by the polymerase causes detachment and separation of the quencher, with consequent fluorescence emission. The reaction was performed on the samples of the Inventors by using the HOT-RESCUE REAL TIME PCR FP Kit (Diatheva). For the ATMdexa1 target a specific probe with Cy3 as reporter fluorophore was
30 designed, while for the housekeeping HPRT1 a specific probe with JOE as

fluorophore was designed, so as to have signals specific for the target gene and the reference gene and be able to carry out both reactions in multiplex PCR. The concentration of the two pairs of primers (Table 1 and 2) was optimized to 500 nM, while the probe concentration was 100 nM. The thermal profile was set
5 on 50 denaturation cycles of 15 sec at 94°C, followed by an annealing/extension step at 60°C for 60 seconds. In this case as well, the quantification of ATMdexa1 can be carried out either in relative manner (with respect to the reference gene) or in absolute manner, as described in the previous example.

10

c) ATMdexa1 can be quantified by a series of other assays, all based on PCR technology. For example, probes to be used in the Molecular Beacons method were designed by the Inventors. In this application, the amplification is performed in the presence of Molecular beacon probes that are labeled at the 5'
15 terminus with a fluorescent reporter dye (FAM for ATMdexa1 and HEX for HPRT1) and at the 3' terminus with a quencher (BHQ1, Tables 1 and 2). During the annealing step, the primers and probes hybridize to the respective sequence of complementary DNA. Hybridization of probes, in particular, causes their "opening" and the entailed separation of the fluorophore and quencher; the
20 quencher therefore can no longer absorb the energy emitted by the reporter fluorophore.

d) Another example of method proposed is based on a Hybridization/FRET probe, consisting in the use of two labeled probes: the first one on the 3' end
25 with a donor fluorophore (FAM), whereas the second one on the 5' end with an acceptor fluorophore (LC red 640).

e) Finally, the PCR assay may be set up from Scorpion technology probes.

The sequences of all the primers pairs and of all the probes proposed in the above-mentioned examples, for amplification and successive quantification of ATMdex1 in the biological samples, are reported in tables 1 and 2. All methods mentioned, as well as any other method set up as variant on the theme of the previous ones, envisaging the quantification of ATMdexa1 by means of polymerase chain reaction (for example, Digital PCR) fall within the scope of the present invention.

10

REFERENCES

1. Broccoletti T, Del GE, Amorosi S, Russo I, Di BM et al. **Steroid-induced improvement of neurological signs in ataxia-telangiectasia patients.** Eur J Neurol 2008, **15**:223-228.
2. Buoni S, Zannolli R, Sorrentino L, and Fois A. **Betamethasone and improvement of neurological symptoms in ataxia-telangiectasia.** Arch Neurol 2006, **63**:1479-1482.
3. Russo I, Cosentino C, Del GE, Broccoletti T, Amorosi S et al. **In ataxia-telangiectasia betamethasone response is inversely correlated to cerebellar atrophy and directly to antioxidative capacity.** Eur J Neurol 2009, **16**:755-759.
4. Broccoletti T, Del GE, Cirillo E, Vigliano I, Giardino G et al. **Efficacy of very-low-dose betamethasone on neurological symptoms in ataxia-telangiectasia.** Eur J Neurol 2011, **18**:564-570.
5. Gatti RA and Perlman S. **A proposed bailout for A-T patients?** Eur J Neurol 2009, **16**:653-655.
6. Biagiotti S, Paoletti MF, Fraternale A, Rossi L, and Magnani M. **Drug delivery by red blood cells.** IUBMB Life 2011, **63**:621-631.

7. Bossa F, Latiano A, Rossi L, Magnani M, Palmieri O et al. **Erythrocyte-mediated delivery of dexamethasone in patients with mild-to-moderate ulcerative colitis, refractory to mesalamine: a randomized, controlled study.** Am J Gastroenterol 2008, **103**:2509-2516.
- 5 8. Castro M, Rossi L, Papadatou B, Bracci F, Knafelz D et al. **Long-term treatment with autologous red blood cells loaded with dexamethasone 21-phosphate in pediatric patients affected by steroid-dependent Crohn disease.** J Pediatr Gastroenterol Nutr 2007, **44**:423-426.
- 10 9. Pierige F, Serafini S, Rossi L, and Magnani M. **Cell-based drug delivery.** Adv Drug Deliv Rev 2008, **60**:286-295.
10. Rossi L, Serafini S, Cenerini L, Picardi F, Bigi L et al. **Erythrocyte-mediated delivery of dexamethasone in patients with chronic obstructive pulmonary disease.** Biotechnol Appl Biochem 2001, **33**:85-89.
- 15 11. Chessa L, Leuzzi V, Plebani A, Soresina A, Micheli R et al. **Intra-erythrocyte infusion of dexamethasone reduces neurological symptoms in ataxia teleangiectasia patients: results of a phase 2 trial.** Orphanet J Rare Dis 2014, **9**:5.

TABLE OF SEQUENCES

	SEQ ID NO: 1:
	ATCTAGATCGGCATTGAGATTCCA
	SEQ ID NO:2
5	GCAGACCAGCCAATTACTAAAC
	SEQ ID NO:3
	CGCCTGATTGAGATCCTGAA
	SEQ ID NO:4
	GTGCCTCAACACTTCTGACCAT
10	SEQ ID NO:5
	AAACATCTAGATCGGCATTGAGATTCCA
	SEQ ID NO: 6
	CGCCTGATTGAGATCCTGAA
	SEQ ID NO: 7
15	GTGCCTCAACACTTCTGACCAT
	SEQ ID NO: 8
	TCTAGATCGGCATTGAGATTCC
	SEQ ID NO: 9
	AGAATGTCTGAGAATAGCA
20	SEQ ID NO: 10
	AACTTAGATGCCACTCAG
	SEQ ID NO: 11
	GATGGTCAGAAGTGTTGAGG
	SEQ ID NO: 12
25	ATCCTGAAACAATTAAACAT
	SEQ ID NO: 13
	ACTTTGTGATGCTTATATTAT
	SEQ ID NO: 14
	TTTACAGAAATATATTCAGAAAGAAACAGA

- SEQ ID NO: 15
TGAGAATAGCAAAACCAAATGTATC
- SEQ ID NO: 16
TATGCTGAGGATTTGGAAAGGGT
- 5 SEQ ID NO: 17
CCATCACATTGTAGCCCTCT
- SEQ ID NO: 18
GGAAAGGGTGTTTATTCCTCATGGA
- SEQ ID NO: 19
- 10 GGCCTCCCATCTCCTTCATC
- SEQ ID NO: 20
TATGGACAGGACTGAACGTCTTGC
- SEQ ID NO: 21
GGATTTGGAAAGGGTGTTTATTCC
- 15 SEQ ID NO: 22
GGCCTCCCATCTCCTTCATC
- SEQ ID NO: 23
TGGACTAATTATGGACAGGACTGA
- SEQ ID NO: 24
- 20 TATGCTGAGGATTTGGAAAGGGT
- SEQ ID NO: 25
CCATCACATTGTAGCCCTCT
- SEQ ID NO: 26
TCGTGATTAGTGATGATGA
- 25 SEQ ID NO: 27
TTATGGACAGGACTGAAC
- SEQ ID NO: 28
TACCTAATCATTATGCTGAGGATT
- SEQ ID NO: 29
- 30 GGCTATAAATTCTTTGCT

SEQ ID NO: 30

GGACATAAAAGTAATTGGT

SEQ ID NO: 31

AGATCCATTCCTATGACTGTAGATT

5 SEQ ID NO: 32

CAGACTGAAGAGCTATTGTAATGAC

CLAIMS

1. A method for evaluating the response of patients affected by Ataxia Talangiectasia to glucocorticoid treatment, comprising a step of qualitative and/or quantitative identification, in the blood of said patients, of the expression of a mRNA variant of the ATM gene (Ataxia-Talangiectasia-Mutated gene), wherein said mRNA variant is produced by non-canonical splicing induced by glucocorticoid selected from the group consisting of prednisolone, dexamethasone, betamethasone, deflazacort, pharmaceutically acceptable salts or esters thereof and mixtures thereof, and contains the Phosphatidyl Inositol 3 Kinase domain, wherein said mRNA variant is amplified using a 5'-3' forward 5'-3' reverse primer pair, wherein detectable expression values of said mRNA variant indicate positive response to treatment, and wherein the 5'-3' forward 5'-3' reverse primer pair is SEQ ID: NO3/SEQ ID: NO4.
2. The method according to claim 1, wherein the glucocorticoid is prednisolone phosphate, dexamethasone phosphate, betamethasone phosphate, deflazacort phosphate or sodium salts thereof.
3. The method according to claim 1 or 2, wherein the mRNA variant of the ATM gene is ATMdexa1 mRNA produced by treatment with dexamethasone or pharmaceutically acceptable salts thereof.
4. The method according to any one of claims 1 to 3, wherein the quantitative identification of the expression of the mRNA variant is normalized versus the expression of a housekeeping gene.
5. The method according to any one of claims 1 to 4, wherein the identification of the mRNA variant is performed by any technique of amplification.

6. The method according to claim 5, wherein the identification of the mRNA variant is carried out by a technique selected from the group consisting of: PCR, RT-PCR, RT-PCR with an intercalating agent, Molecular Beacon probe method PCR, FRET-probe hybridization PCR and Scorpion probe PCR.

7. The method according to claim 6, wherein the detection of amplification is carried out using a probe selected from the group consisting of SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:14 and SEQ ID NO:15.

8. The method according to any one of claims 5 to 7, wherein the glucocorticoid is a glucocorticoid encapsulated within erythrocytes.

9. The method according to claim 8, wherein the glucocorticoid is dexamethasone phosphate mono or disodium.

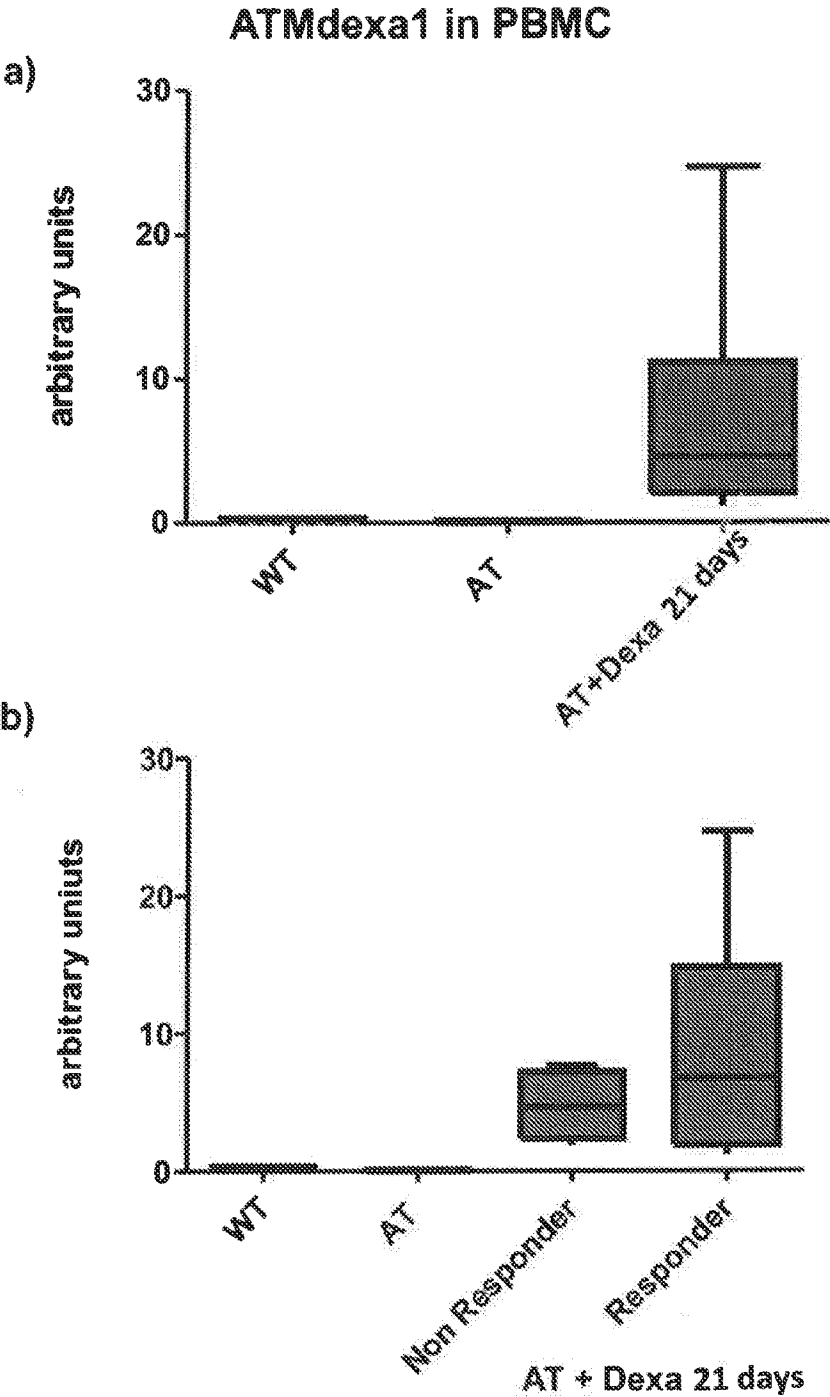
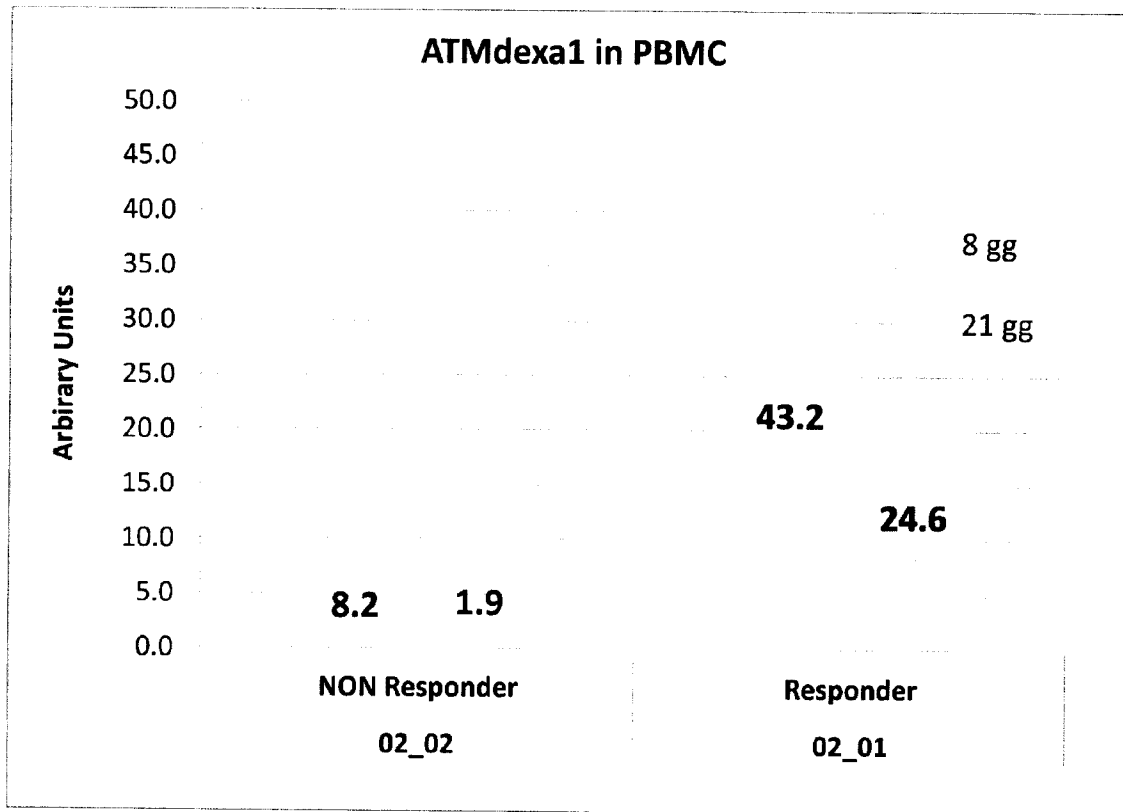
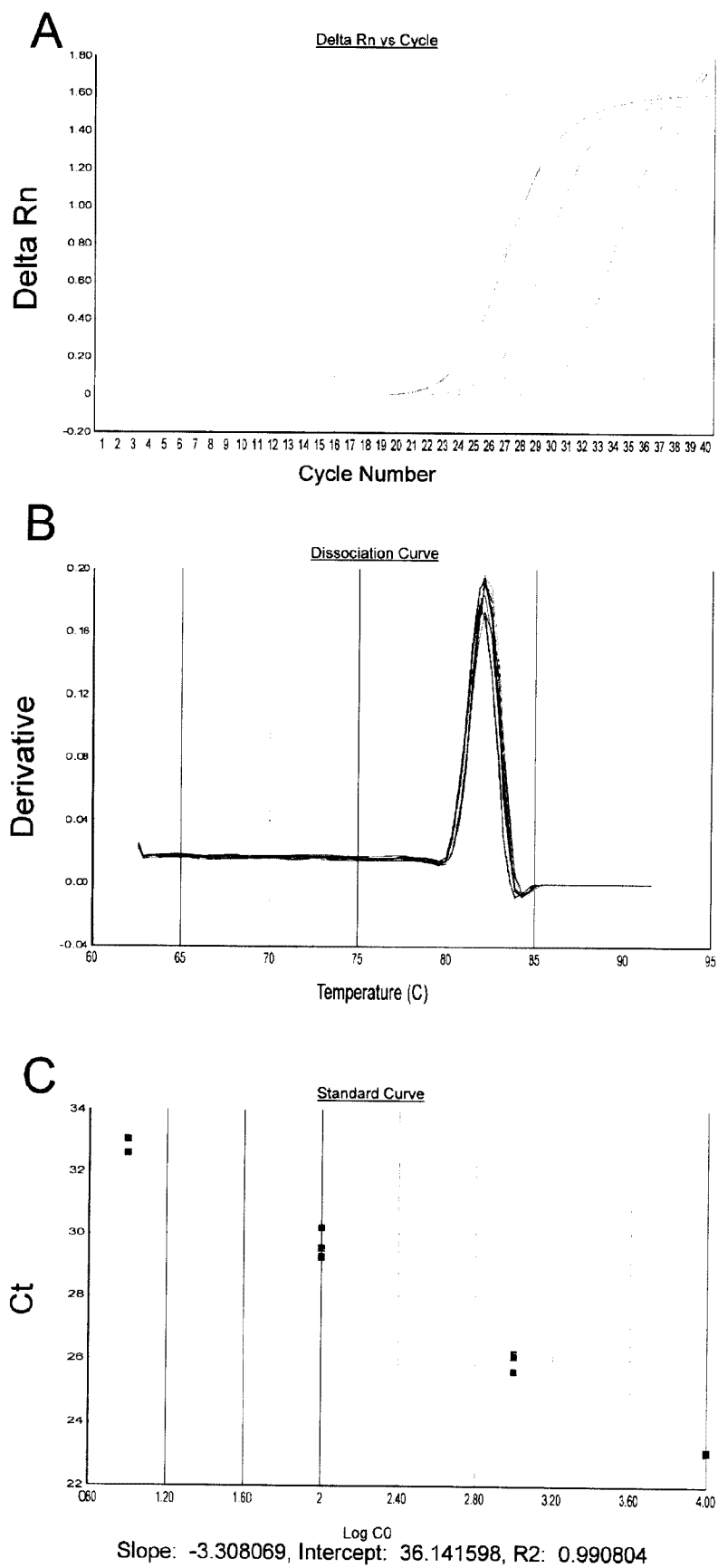
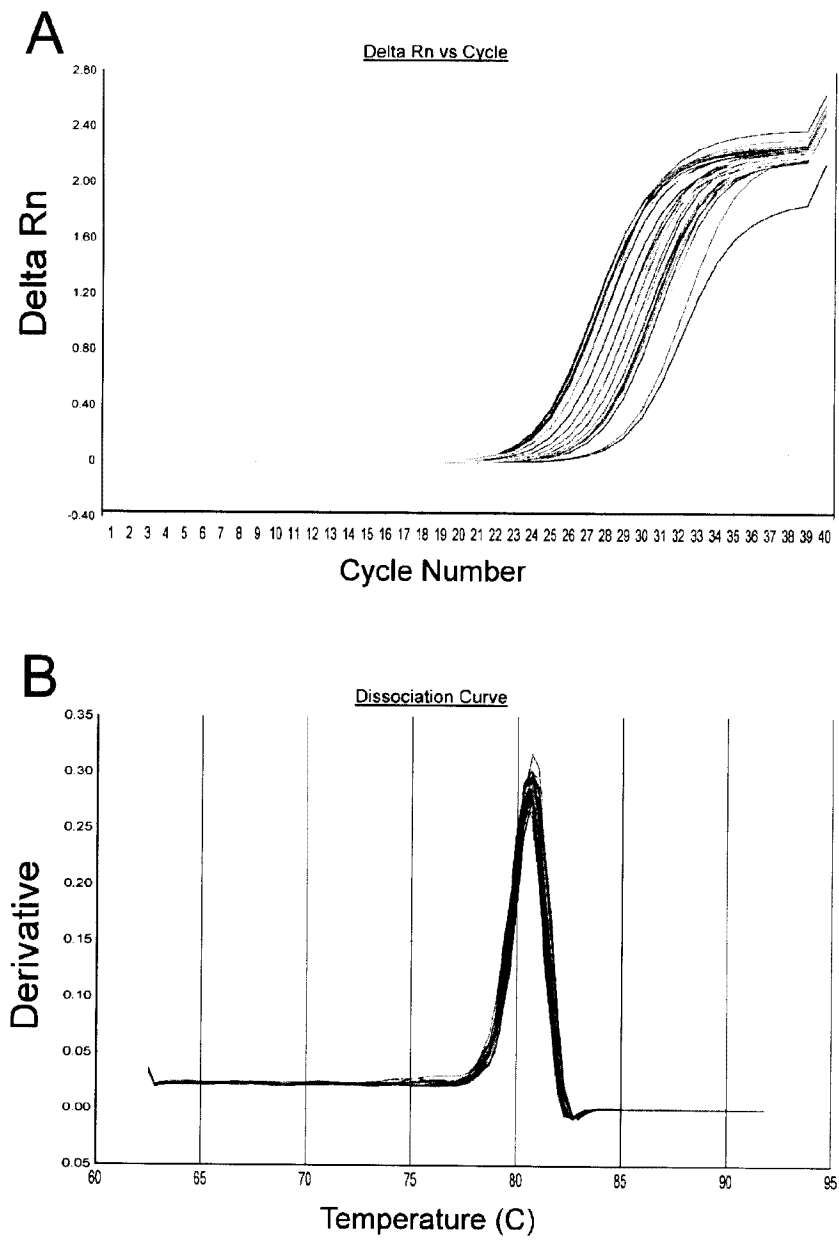


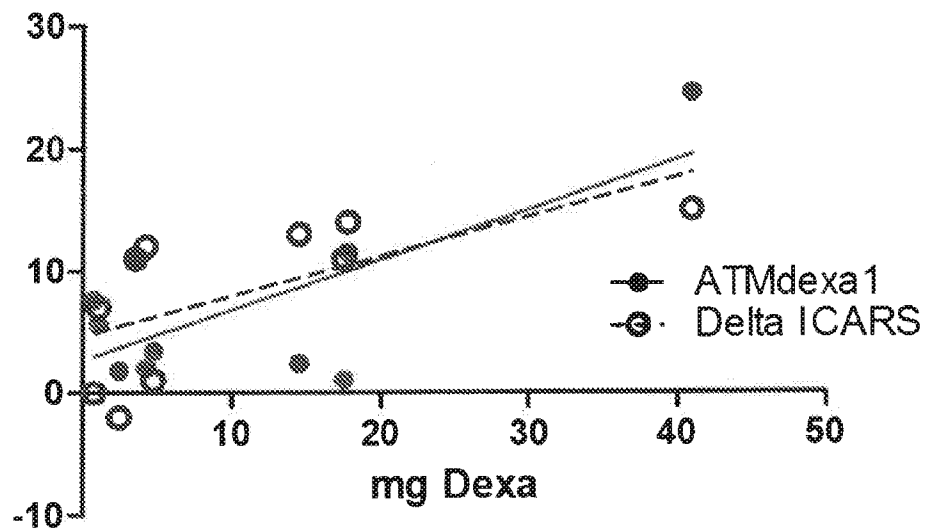
FIGURE 1

**Figure 2**

**Figure 3**

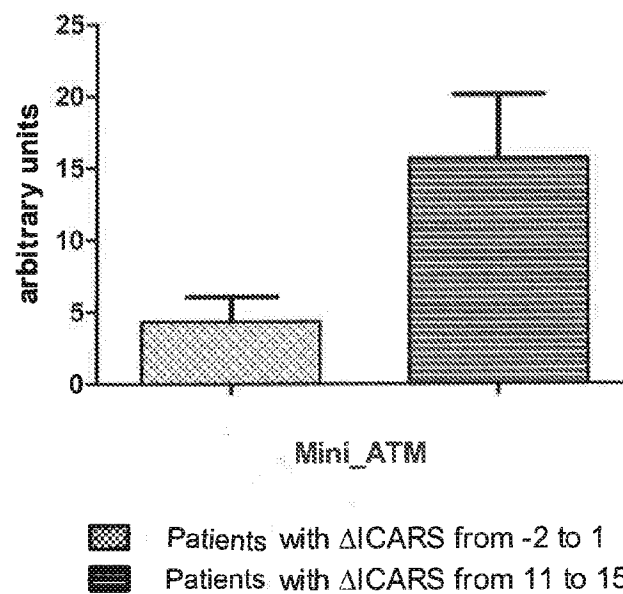
**Figure 4**

a)



	ATMdexa1	Delta ICARS
Number of XY pairs	10	10
Pearson's r	0.7136	0.6466
95% confidence interval	0.1523 to 0.9268	0.02848 to 0.9070
(two-tailed) P value	0.0206	0.0433
P value summary	*	*
Significant correlation? (alpha=0.05)	Yes	Yes
R ²	0.5092	0.4181

FIGURE 5a



Number of values	3	3	3	3
Mean	4.317	-0.3333	15.69	13.33
Standard deviation	3.003	1.528	7.713	2.062
Standard error	1.734	0.8819	4.453	1.202
Lower 95% CI of mean	-3.142	-4.128	-3.470	8.162
Upper 95% CI of mean	11.78	3.461	34.85	18.50
Total	12.95	-1.000	47.07	40.00

1 mini ATM	1 Δ ICARS	2 Mini ATM	2 Δ ICARS
3.44	1.	10.95	11.
7.66	0.	11.53	14.
1.85	-2.	24.59	15.
Mini ATM	cut-off = 4.3 ± 6		
Δ ICARS	cut-off = -0.33 ± 3		

FIGURE 5b

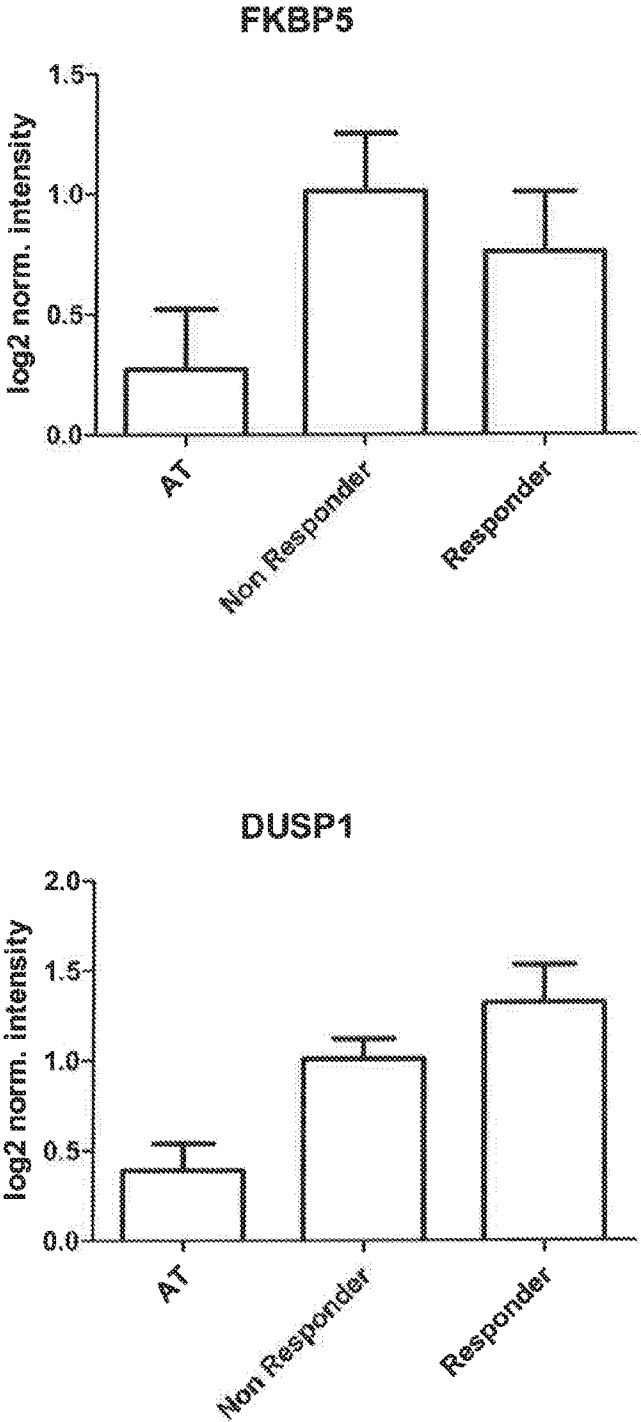


FIGURE 6

ATMdexa1 in PBMC

