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Treatment of Dexamethasone in A-T patients derived cell lines: investigation of the drug effects in different ATM signalling pathways.

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Summary

Chapter 1
Introduction

Ataxia-Telangiectasia.

Ataxia-Telangiectasia (A-T, MIM # 208900) is a rare autosomal recessive disease affecting one child in 40,000-300,000 live births (Chun and Gatti, 2004). The disease is caused by mutations in the Ataxia-Telangiectasia Mutated (*ATM*) gene that generate a non-functional ATM protein.

A-T patients exhibit a pleiotropic phenotype that includes progressive cerebellar degeneration, telangiectasia, immunodeficiency, recurrent sinopulmonary infections (in about 60-80% of patients, starting at age of three), radiation sensitivity, premature aging, and a predisposition to cancer development, especially of lymphoid origin (McKinnon, 2009; Boder and Sedgwick, 1958). Other abnormalities include growth delay, gonadal atrophy, delayed pubertal development and insulin resistant diabetes (Nissenkorn et al., 2016). Cells derived from A-T patients are more sensitive to ionizing irradiation, present chromosomal instability, shortened telomeres, premature senescence and a defective response to DNA double strand breaks (DSBs) (Shiloh et al., 2001; Shiloh et al., 2003; Derheimer et al., 2010).

Clinical manifestation of A-T patient.

Cerebellar ataxia usually appears after the first year of life, with the onset of walking.

This symptom occurs with the loss of coordination of voluntary movements of legs and arms, progressively degenerating in a generalized motor dysfunction of the limbs, ocular movements, and language. The disease is named after telangiectasia, small capillary dilated mostly in the sclera of the eye and on the skin of many A-T patients. Ataxia is progressive and generally lead patients to wheelchair around the second decade of life (Crawford et al., 1998). In the meantime, they show impaired coordination of eye movement (nystagmus) and dysarthria.

The neurological symptoms are related to a diffuse degeneration and atrophy of the cerebellar vermis and hemispheres, involving Purkinje cells (PCs) and, to a lesser extent, granule neurons. Various neuropathological abnormalities (e.g., neuronal changes, gliosis and vascular changes) have also been observed in the cerebellum, brain stem and spinal cord (Boder et al., 1985; Sahama et al., 2014).

Telangiectasia is the second major clinical symptom that occurs later than ataxia usually between 2 and 8 years of age. This is due to dilation of blood vessels, within the bulbar conjunctiva over the exposed sclera of the eyes.

About two-thirds of people with A-T have immunodeficiency (Nowak et al., 2004; Driessen et al., 2013). The most common abnormalities are low levels of one or more classes of immunoglobulin (IgG, IgA, IgM or IgG subclasses), failure to make antibodies in response to vaccines or infections, and lymphopenia, especially affecting T-lymphocytes. Recurrent infections are a major clinical feature of A-T with up to 80% of patients being affected (Schroeder et al., 2014; Lavin et al., 2017). These include both upper and lower respiratory tract infections associated with the development of bronchiectasis or persistent pleural abnormalities (Crawford et al., 2006).

The second most common cause of death in patients with A-T is cancer with a lifetime prevalence of 30% which is approximately 100-fold greater than expected for an age-matched population. Lymphoma and leukemia most often occur in A-T patients before the second decade of life (Suarez et al., 2015).

The progressive aggravation of neurological and immunologic symptoms, mostly due to chronic lung disease, leads patients to death within the second decade of life. In 2006, the average life expectancy was reported to be approximately 25 years (Crawford et al., 2006).

ATM: a member of the PIKK family.

ATM is located on human chromosome 11q22-23 and is made up of 66 exons (four non-coding and 62 coding) spanning 150 kb of genomic DNA. *ATM* encodes for a large protein of 350 kDa comprising 3,056 residues. Its prominent role is coordinating the cellular response to DNA damage. However, in the past years, different studies have deeply investigated ATM function in response to oxidative and genotoxic stresses that affect cellular homeostasis, resulting in the direct phosphorylation and regulation of an ever-growing list of downstream substrates (Paz et al., 2011; Matsuoka et al., 2007)(Table 1).

Table.1. Summary of the ATM protein features.

- 3056 amino acids
- Serine/Threonine protein kinase
- Member of the family of PI3 Kinase-like Kinases (PIKKs)
- Located primarily in the nucleus; smaller amounts in the cytoplasm and associated with mitochondria and peroxisomes [178]
- Activated primarily by DSBs and oxidative stress, but also agents affecting chromatin organization, hypoxia, hypotonic stress and hyperthermia
- Phosphorylates and regulates a variety of protein substrates involved in
  - The DNA damage response (NHEJ and HRR) to DSBs
  - Various other genotoxic stress responses
  - DNA repair processes
  - Cell cycle checkpoints
  - Other cell stress responses
  - Apoptosis

NHEJ non-homologous end-joining, *HRR* homologous recombination repair

The carboxyl-terminal active site is the landmark domain of ATM. This domain places ATM within the family of PI3K like protein kinases (PIKKs), most of which are involved in cellular responses to various stresses (Lovejoy et al., 2009). The PI3K domain spans approximately 10% of the ATM protein. The remaining protein contains regulatory and interaction domains that determine its modes of activation and broad substrate specificity, although they are not fully understood (Lempiainen et al., 2009).

Other members of the PIKKs family are ATR (ATM and RAD3related), DNA-PK (DNA-dependent protein kinase catalytic subunit), SMG1 (suppressor of mutagenesis in genitalia 1), mTOR (mammalian TOR) TRRAP (transformation/transcription domain-associated protein that lacks catalytic activity) and AKT. The PI3K-like domain and two other C-terminal domains, FAT (conserved in FRAP, ATM and TRRAP) and FATC (FAT C-terminal), are shared among PIKKs (Fig. 1).

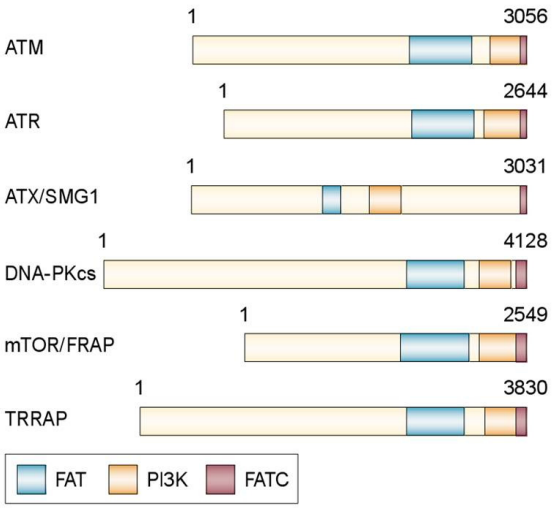


Figure 1. ATM and other PIKKs (Shiloh et al., 2003). The image represents all known ATM orthologues and shared domains. All these proteins are involved in the response to different cellular stresses and they act by phosphorylating various substrates.

Regulation of DNA damage response.

The double strand break (DSB) response is an extremely important and calibrated response that weaves together various complex signaling pathways in order to maintain genomic integrity across organisms and prevents any unwanted/unsustainable alterations in the genetic pool. DSBs are considered the most important determinant of cell fate following DNA damage induced by irradiation or free radicals. Within minutes of occurrence of DSBs, mammalian cells accumulate a plethora of repair and signaling proteins at the site of damage. This complex response to double strand breaks, involving multiple cellular pathways, is regulated by ATM in a prominent way. This intricate cellular response works in a hierarchical manner through the transduction of messages from 'sensors' through 'transducers' to the various 'effector' molecules.

Various PIKKs are activated in response to different forms of damage: ATM and DNA-PK mediate the response to double strand breaks, whereas ATR responds to single strand breaks. The DSB damage is sensed by the MRN complex (Meiotic Recombination Protein-11 (MRE-11)-Rad50-Nijmegen Breakage Syndrome-1(Nbs1)) complex for ATM, whereas ATR and DNA-PK are recruited to the site of damage by ATRIP and Ku80 proteins.

ATM is recruited as an inactive dimer to the site of double strand break. ATM molecule phosphorylates its counterpart within the ATM dimer at the serine-1981 position in the FAT domain. This phosphorylation causes the dissociation of ATM dimer turning them into kinase-active monomers (Fig. 2).

Once ATM is recruited to the site of DNA damage it phosphorylates histone variant H2AX at serine-139 residue at the DNA double strand break to form γ-H2AX (Rogakou et al., 1998), that accumulates at the damage site and is seen as discrete nuclear foci starting as early as one minute after irradiation, with maximal phosphorylation seen by 10–30 min (Rogakou et al., 1999). The phosphorylation of H2AX is followed by recruitment of MDC1 (Mediator of DNA Damage Checkpoint Protein 1). MDC1 contains Fork Head Associated (FHA) domain at its N-terminus and BRCA1 carboxyl-terminal repeat (BRCT) motifs at its C-terminus. MDC1 interacts with the phosphor-domain of γH2AX through its BRCT domain, forming foci, which overlap with that of H2AX foci. In addition, MDC1 helps in the retention of ATM at the damage site via direct interaction with the MRN complex and the subsequent binding of ATM to the C-terminus of NBS1 (Stucki et al., 2005).

Once activated, ATM also controls cell-cycle–checkpoint signaling, perhaps to maintain a clear temporal separation between DNA repair and cell division, which probably should not be happening at the same time. ATM coordinates: (1) the G1-S cell-cycle checkpoint by phosphorylating the tumor suppressor protein p53 at Ser15; (2) the G2/M checkpoint by phosphorylating the protein kinase Chk2 at Thr68; and (3) the intra-S cycle checkpoint by phosphorylating SMC1 at Ser957 and Ser966 (Kurz et al., 2004; Abraham et al., 2001; Yazdi et al., 2002). The role of ATM in maintaining genomic stability by coordinating the DNA-damage response and regulating cell-cycle damage checkpoints is consistent with it being most abundantly expressed in the nucleus.

DNA-PK is the catalytic subunit of the DNA-PK holoenzyme, which also contains the KU70–KU80 heterodimer. It has a central role in the non-homologous end-joining (NHEJ) DSB repair pathway, but its involvement in other processes, such as cell proliferation and regulation of oxidative stress during inflammation, is being noted (Hill et al., 2010).
ATR has a major role in coordinating DNA replication origin firing and guarding replication fork stability, and its canonical pathway activates a DNA Damage Response (DDR) network following replication fork stalling, which may lead to subsequent collapse (Cimprich et al., 2008).

The primary stimuli, modes of activation and interacting proteins of the three PIKKs are different (Kong et al., 2011; Chen et al., 2011). Nevertheless, there are overlaps in their targets, functional crosstalk and even collaboration among them following certain genotoxic stresses, depending on the type and extent of the damage and the time point after damage induction (Serrano et al., 2012; Stiff et al. 2006). This delicate balance and cooperation are abrogated by the loss of ATM in patients with Ataxia-Telangiectasia. Interestingly, cells from patients with A-T show many DDRs that are considered ATM dependent, but that are markedly attenuated compared with wild-type cells (Serrano et al., 2012). It is possible that the absence of ATM affects the responses of DNA-PK and/or ATR to DSBs, and their collaborative activity is stimulated to replace partially the missing member of the trio.

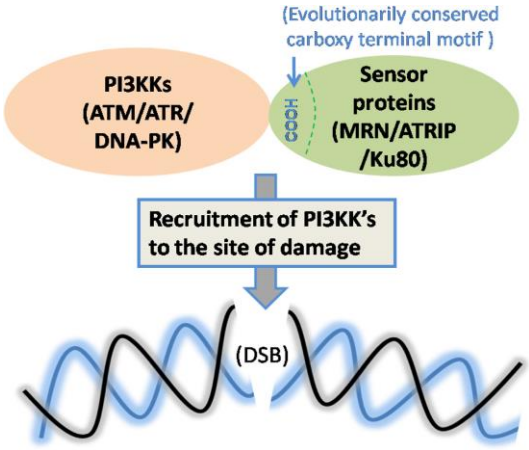


Figure 2. Schematic representation of conserved mode of recruitment of PI3KK's to the site of DNA damage. Sensor proteins; MRN complex (Mre11/Rad50/Nbs1) complex, ATRIP and Ku80 proteins mediate the recruitment of ATM, ATR and DNA-PK respectively to the site of damage via interaction with evolutionarily conserved carboxy terminal motif of these sensor proteins.

ATM and oxidative stress.

Many studies have suggested the involvement of ATM in regulating the global cellular response to oxidative stress. Using ATM-deficient cell lines and tissues, several authors confirmed the involvement of ATM in oxidative stress signaling (Kamsler et al., 2001; Takao N. et al., 2000), although the molecular mechanisms underlying ATM roles are still elusive (Barzilai et al. 2002; Reliene et al., 2004; Kamsler et al., 2001; Chen et al, 2003; Takao et al., 2000). Two primary hypotheses on how the ATM deficiency leads to oxidative stress advocate direct or indirect involvement of ATM in the phenomenon (Barzilai et al 2002; Reliene et al., 2004).

According to the first hypothesis, ATM may directly modulate the redox environment of a cell by maintaining optimum levels of antioxidants, either by regulating the genes encoding these antioxidant molecules or by modifying antioxidant enzymes (Barzilai et al., 2002). Regulation of antioxidants by ATM is further suggested as A-T cells have low levels of various antioxidants and diminished glutathione synthesis (Reichenbach et al., 1999; Dean et al., 1988). ATM was also shown to have extra nuclear localization in peroxisomes, a major site of oxidative metabolism (Watters et al., 1999), and this further strengthens the role of ATM in direct regulation of oxidative stress signaling. The other dimension of this direct control suggests that ATM can sense and respond directly to abnormal levels of ROS. Very recent experimental evidence has suggested that ATM acts as a sensor in the oxidative stress-signaling cascade by being directly activated by oxidation (Guo et al., 2010).

An alternative hypothesis posits indirect involvement of ATM in regulation of oxidative stress. In this idea, ATM deficiency leads to persistent DSBs that could be otherwise repaired in normal cells. This persistent DNA damage leads to activation of repair enzymes such as PARP, which synthesizes PAR chain at the site of damage. The substrate for generating PAR is NAD+ and hence this process depletes the stores of cellular NAD+, reducing the antioxidant capacity of cell. Therefore, increased ROS can be an outcome of persistent DNA damage existing in ATM-deficient cells (Barzilai et al., 2002; Reliene et al., 2004).

These studies proposed that, similar to DNA damage response, ATM may regulate global cellular responses to oxidative stress. Guo et al. (2010) suggested that oxidants activate ATM through different biochemical mechanisms than that of MRN complex signaling, which is well known in the DNA damage response pathway. When primary human fibroblasts were treated with H_2O_2, autophosphorylation of ATM was observed at serine-1981 together with phosphorylation of its substrates, whereas no phosphorylation or activation was observed in the case of H2AX. This observation led to the conclusion that H_2O_2 can activate ATM even in the absence of DNA damage, thus showing that ATM may be acting as an oxidative stress sensor. A summary on the role of ATM in oxidative stress is reported in fig. 3.

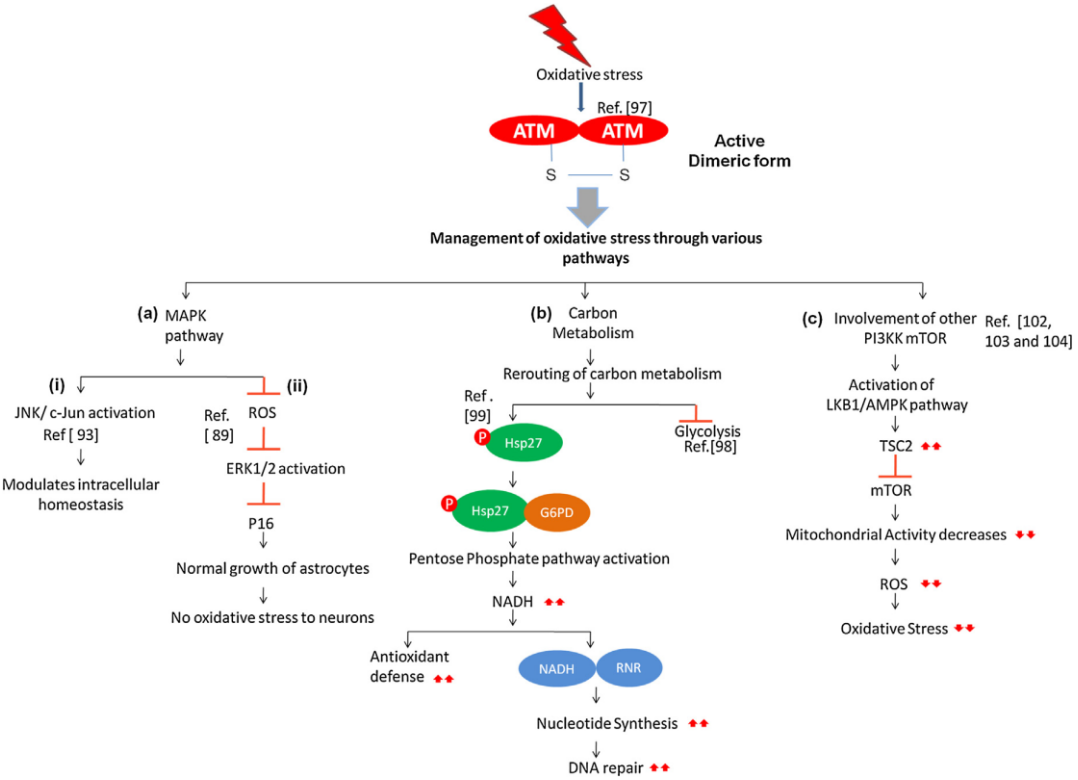


Figure 3. ATM and oxidative stress (from Guleria and Chandna, 2016).

In response to oxidative stress, ATM forms kinase active covalent homodimers through intermolecular disulphide bonds and manages oxidative stress via various pathways. (A) Via MAPK pathway. (i) ATM in response to oxidative stress activates JNK-c jun pathway and thus modulates intracellular redox homeostasis. (ii) ATM inhibits ERK1/2-p16 pathway, resulting in normal growth, proliferation and maturation of astrocytes, which support neurons under oxidative stress. (B) Via rerouting of carbon metabolism. Under stress conditions, ATM reroutes the metabolic flux from glycolysis to the pentose phosphate pathway (PPP). ATM mediates this metabolic shift by promoting the phosphorylation of Hsp27 that in turn binds to G6PD, the limiting enzyme of PPP, thereby stimulating its activity. NADH produced as a product of PPP helps the cell in mounting antioxidant response and enhancing nucleotide synthesis and thus DNA repair under stress conditions. (C) Involvement of mTOR in oxidative stress signaling. Under oxidative stress, ATM represses mTOR via activation of the LKB1/AMPK pathway. This consequently inhibits mitochondrial activity and ROS levels thus protecting cells from oxidative stress.

Cytoplasmic ATM functions.

Other sequences identified within ATM include several nuclear localization signals, a leucine zipper and a HEAT repeat sequence (Abraham et al., 2001), a motif found in many nuclear and cytoplasmic proteins involved in protein trafficking. Although many functions of ATM have been deduced from its amino acid sequence, corresponding laboratory studies have not yet identified functions for >90% of the remaining sequence. This indicates that ATM could have many, yet unknown, additional functions (Shiloh et al., 2001).

However, many other physiologically relevant symptoms associated with A–T, such as insulin resistance and neuronal degeneration, are difficult to explain by the lack of DNA damage control. Although ATM has been traditionally considered a nuclear protein that functions in response to genotoxic stress, there is increasing evidence suggesting it also has separate cytoplasmic functions (Abraham et al., 2001; Fig. 4). In fact, in neuronal or neuron-like cells ATM localization was found to be predominantly cytoplasmic (Oka et al., 1998; Boehrs et al., 2007). Another study revealed that a fraction of ATM is found in the cytoplasmic compartments of proliferating cells where it associates with b-adaptin, a cytoplasmic protein involved in vesicle trafficking (Lim et al., 1998). More importantly, clues about the cytoplasmic function of ATM have come from the discovery of its involvement in insulin signaling pathways (Yang et al., 2000; Halaby et al., 2008).

In the first report demonstrating a role of ATM in insulin signaling, ATM kinase activity was found to increase 3-fold in response to insulin in rat 3T3-L1 cells that had differentiated into adipocytes (Yang et al., 2000; Viniegra et al., 2005). In addition, insulin leads to phosphorylation of 4E-BP1 (also called PHAS-I), an insulin-responsive cytoplasmic

protein, in an ATM-dependent manner. Phosphorylation of 4EBP1 at Ser111 by ATM promotes initiation of mRNA translation (Yang et al., 2000). More recently, it was discovered that ATM stimulates insulin induced AKT phosphorylation at Ser473 and mediates the full activation of AKT activity (Halaby et al., 2008). AKT participates in multiple physiological processes, including protein translation, glucose uptake, cell proliferation and cell survival, in response to insulin and many other growth factors. Therefore, these findings open the door for exploring the unknown functions of ATM and could provide explanations for many of the clinical phenotypes of A-T that are difficult to explain by the nuclear localization and functions of ATM (Yang et al., 2011).

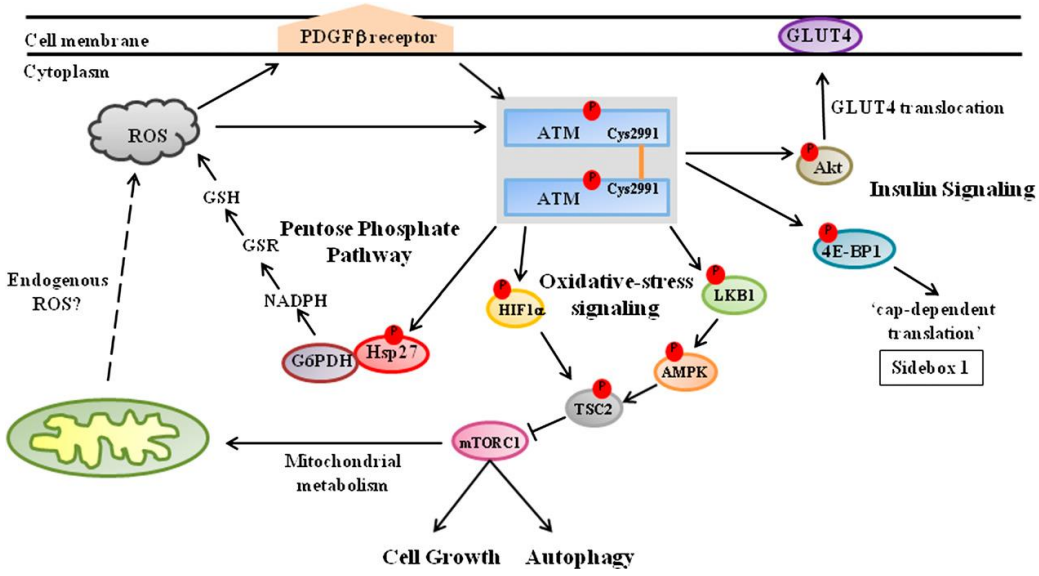


Figure 4. ATM kinase signals to diverse metabolic pathways in the cytoplasm. During in vitro experiments, oxidants/pro-oxidants induce the phosphorylation and activation of ATM dimers that appear to be linked via intermolecular disulfide bonds formed at a conserved C2991 residue in the C- terminus (Guo et al., 2010). It remains to be fully clarified whether these ATM dimers are similarly activated in response to endogenously generated ROS-acting directly on ATM, or else via the platelet-derived growth factor receptor b (Kim et al., 2010)-and are responsible for regulating (1) expression of mTORC1; (2) synthesis of reduced glutathione (GSH) via the PPP; and (3) insulin-induced protein synthesis in response to oxidative stress.

Genotype / phenotype correlations.

A-T patients can be roughly divided into two groups: the “classic” or “early-onset” patients who show the severe neurodegenerative phenotype, and the “mild” or “late onset” patients who show milder forms of the disease. In the majority of the patients, the severity of the clinical phenotype correlates with the mutation type found in the *ATM* gene.

The *ATM* gene is one of the largest genes in the genome with 66 exons (four in the 5'-UTR and 62 in the coding region) spanning 150 kb of genomic DNA (Telatar et al., 1998) and has no hot-spots mutations. These include primarily nonsense and frame-shift mutations resulting from insertions and deletions, but also missense and leaky splice-site mutations. The majority of the patients are compound heterozygous (Hassin et al., 1998).

Truncating mutations lead to a highly unstable protein, which is usually not detectable by western blot analysis and has not kinase activity. Individuals who carry these type of mutations have a classic clinical presentation of A-T, and the severity of their disease follows a relatively predictable course (see Fig. 5).

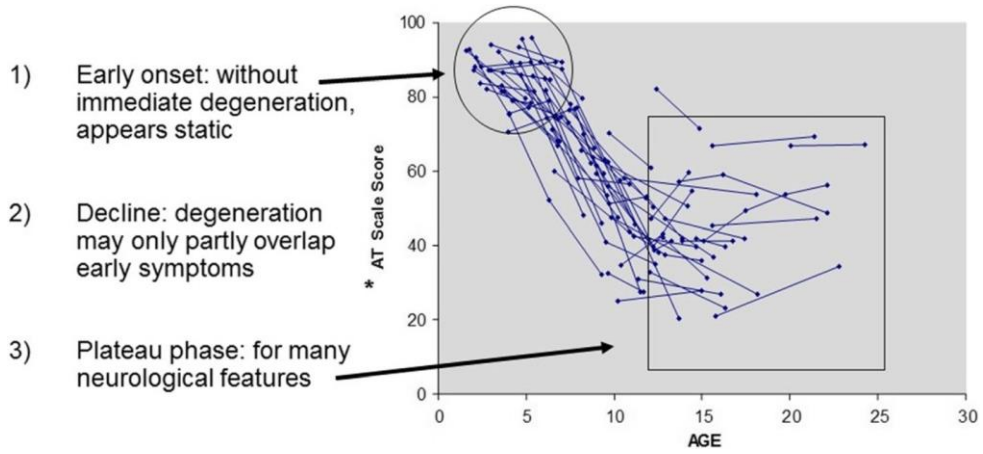


Figure 5. The pattern of neurological decline associated with the classic presentation of A-T (Oviatt et al., 2016).

Missense, in-frame or leaky splice-site mutations allow for the production of residual amounts of functioning ATM protein (Taylor et al., 2015) detected by western blot. This has residual kinase activity. Individuals with these types of mutations show a milder A-T phenotype due to the presence of a residual functional protein, that lead to a less severe phenotype and a slow progression of the neurological symptoms.

However, some exceptions have been reported (Worth et al., 2013; Alterman et al., 2007). A-T patients with null *ATM* mutations (frameshift and splice site mutations causing truncation), no ATM protein detectable by western blot, no kinase activity and the typical cellular A-T phenotype for classic A-T showed slow disease progression. One possible explanation is a still unknown compensatory mechanism. Although rare, these patients are of particular interest because the genetic and/or environmental factors that modify the severity of their clinical course may provide hints for treatment interventions.

ATM and neurodegeneration.

Assuming the prominent role of ATM as a sensor of DSBs, it is challenging to correlate its loss-of-function with the complex phenotype of the disease. The most devastating symptom of A-T is progressive cerebellar degeneration, characterized by the gradual loss and/or aberrant location of PCs and, to a lesser extent, the gradual loss of granule cells (Shanbhag et al., 2010; Pankotai et al., 2012). The reason why these cells are more susceptible than others to ATM loss is unknown, though many hypotheses have been proposed (Hoche et al., 2012) (Table 2).

Table 2. Hypotheses to explain the neurodegeneration in A-T (Hoche et al., 2012).

- Defective DDR [183, 184] or repair resulting in:
  - the failed clearance of genomically damaged neurons during development [76, 185]
  - transcription stress [119] and abortive transcription involving topoisomerase 1 cleavage complex (TOP1cc) dependent lesions [186–189]
  - aneuploidy [190]
- Defective response to oxidative stress characterized by elevated ROS and altered cellular redox status [191–194] and reviewed in [11, 195, 196]
- Mitochondrial dysfunction [197–199] and reviewed in [11]
- Defects in neuronal function involving:
  - Failed cell cycle regulation resulting in the re-entry of post-mitotic (mature) neurons into the cell cycle [200]
  - Synaptic/vesicular dysregulation [201–203]
  - Altered epigenetics including
    - HDAC4 nuclear translocation [204]
    - Histone H3 hypermethylation [205] and
    - Reduced 5-hydroxymethylcytosine [206]
- Defects in brain vasculature [207]
- Altered protein turnover [208]

DDR DNA damage response

A defective response to genotoxic and/or oxidative stress could contribute to the neuronal cellular dysfunction and cell death in A-T. However, a combination of the mechanisms listed in Table 2 is likely to contribute to neuronal cell death in the absence of ATM. Noteworthy, the loss of Purkinje cells cannot explain all of the neurologic abnormalities of A-T patients and the effects of ATM deficiency on other areas of the brain outside the cerebellum are being actively investigated.

ATM and pulmonary infections: failure of the innate immune response.

Despite the cerebellar ataxia is the most prominent feature of A-T, lung failure is one of the more life-threatening symptoms that severely predispose to recurrent sinopulmonary infections of the upper-respiratory tract and oral tissues (McGrath-Morrow et al., 2008). It is well known that bacterial pneumonia and chronic lung disease have been a major cause of death in A-T patients (McGrath-Morrow et al., 2010). These infections have been associated to adaptive immune defects derived by the role of ATM in DNA repair during immunoglobulin and T-cell receptor gene rearrangements. However, patients with clinical manifestations of cell-mediated immune-deficiencies are rare and predisposition to such infections often do not correlate with deficiencies in antibody responses (McGrath-Morrow et al., 2010). Thus, there is no convincing explanation for the reason(s) why some A-T patients are more susceptible to lung failure than others, and if this defective response is due to an impairment in the adaptive or in the innate immune response.

Bacteria have pathogen-associated molecular patterns (PAMPs), highly conserved structural motifs within a microbial species. After cell infection, PAMPs are recognized as non-self by immune cells such as leukocytes through "pattern-recognition receptors" (PRRs). Similarly, cellular injury can release endogenous damage-associated molecular patterns (DAMPs) that can also activate the innate immune system (Janeway et al., 2002). The innate inflammatory immune response is of crucial importance for the early containment of infection but, at the same time, has the potential to result in immunopathology.

One important class of PRRs in the recognition of PAMPs and DAMPS in inflammation are Toll-like receptors (TLRs), which are crucial in the initiation of the host inflammatory response. Other receptors, for sensing microbial infection, include secreted mannan-

binding lectin, surface expressed macrophage scavenger receptor and the intracellular nucleotide-binding oligomerization domain. Following detection of a PAMP or a DAMP, resident inflammatory cells, such as macrophages and dendritic cells, secrete inflammatory cytokines, such as IL-6, COX-2, TNF-α and IL-8. These recruit other immune cells, such as neutrophils, to induce the synthesis of adhesion molecules on endothelial cells at the sites of inflammation (Janeway et al., 2002). Circulating leukocytes then adhere to the endothelium allowing them transmigrating to the infected tissue and aiding with pathogen eradication. Other than the professional immune cells, fibroblasts, as active contributors to the regulation of the inflammatory response, provide the first barrier against pathogens (Silzle et al., 2004).

Triggering of PRRs by microbial or endogenous danger signals culminates in transcriptional induction of cytokine genes and the activation of inflammatory caspases (Inflammasome). Together, these coordinate the synthesis and post-translational processing of cytokines and other effector molecules essential for anti-microbial innate immunity and subsequent adaptive immunity (Kumar et al., 2011).

Nuclear factor-κB (NF-κB) is a major transcription factor that plays a key role in inflammation, apoptosis, and oncogenesis. Moreover, its activation triggers both the innate and adaptive immune response (Kumar et al., 2011). The NFκB family encompasses five members, that act in homo-or heterodimers: p65 (RelA), RelB, c-REL, NFκB1 (p50/p105) and NFκB2 (p52/p100).

The most studied is the p65-p50 heterodimer, where p50 increases DNA binding and p65 confers transcriptional regulation. The quiescent p65-p50 dimer is in the cytoplasm, held by an inhibitory IκB molecule. When a pro-inflammatory signal, such as TNFα, reaches the cell, the subsequent phosphorylation and ubiquitination of IkB leads to the nuclear translocation of NFκB (see fig.6). NFκB binds to “NFκB specific promoter” recognition sites and enhances gene expression of multiple pro inflammatory genes. The NFκB is subject to a negative feedback loop as it stimulates the transcription of IκB.

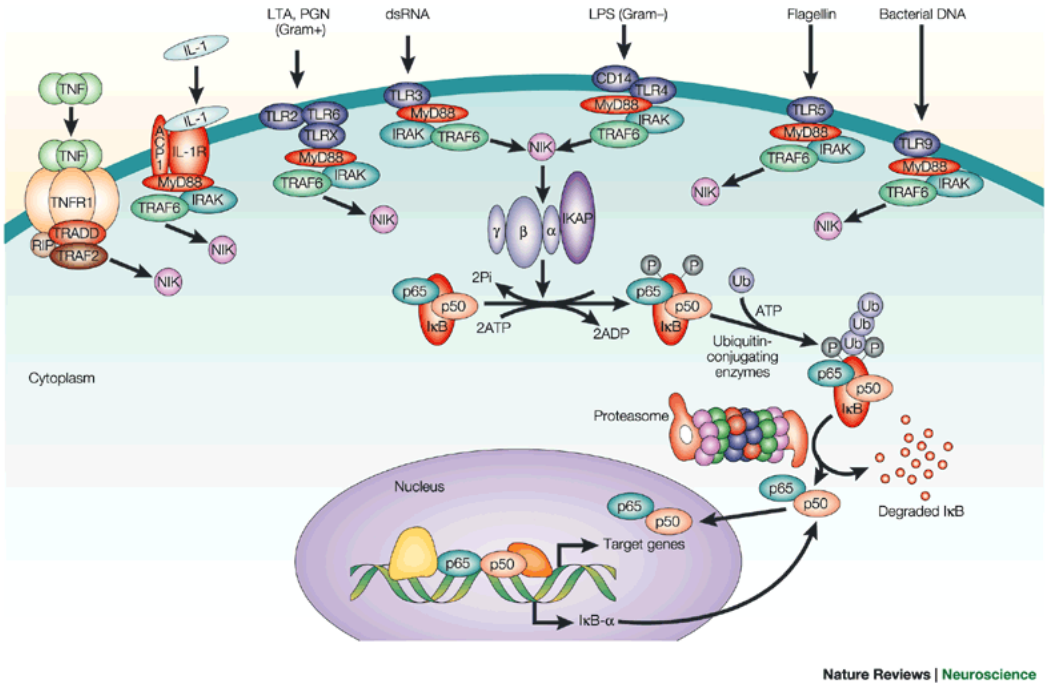


Figure 6. The family of TLRs and pro-inflammatory signal-transduction pathways that recruit NFκB (Nguyen et al., 2002). Inactive NF-kB is retained in the cytoplasm bound to inhibitor of NF-kB (IkB) family members. Upon activation through TNF-α dependent phosphorylation and ubiquitin-mediated degradation of the IkB proteins, NF-kB translocates to the nucleus and upregulates a panel of proteins including pro- and anti- apoptotic cytokines. Deficiencies in NF-kB activation or inhibition of new protein production render a cell extremely sensitive to inflammatory stimuli.

The activation of NFkB during inflammation and microbial infection is mediated by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Triggering of PRRs by microbial or endogenous danger signals culminates in transcriptional induction of cytokine genes, as well as the activation of the inflammasome leading to the release of IL1β, resulting in efficient anti-microbial innate immunity (Nguyen et al., 2002). All these proteins have the activation of the transcription factor NF-κB, as final effector. A direct role of ATM in controlling the activation of the NF-κB pathway has been reported in response to various stimuli, such as cytokines, mitogens, oxidants and other stresses like ionizing radiations (Erttmann et al., 2016). The discovery of a defect in NF-κB activation pathway in A-T cell lines supports the idea that ATM deficiency is associated with primary defects in the innate pathway, affecting both the magnitude and kinetics of the NF-κB response (Fang et al., 2014). This may explain the development of a state of chronic inflammation, likely playing a direct role on the progression of pulmonary disease and on neurodegeneration (Lavin et al., 2016).

Toll-like receptors (TLRs) act as essential sensors of pathogen-associated molecular patterns, ranging from lipopeptide to nucleic acids. TLRs are cell surface PRRs that belong to the type I transmembrane receptor family and are expressed on many cell types including neutrophils, monocytes and fibroblasts. They are characterized by an extracellular leucine-rich repeat domain and an interior Toll/IL-1 receptor (TIR) domain.

Nine of the ten human TLRs are functional and can be found either on the surface of cells (TLR 1, 2, 4-6), where they recognize microbial membrane components, or intracellularly (TLR 3, 7-9), where they recognize microbial nucleic acids.

Lipopolisaccarides (LPS) from *E.coli* are one of the bacterial peptides identified by TLRs family (TLR-4) (Park et al., 2009). First LPS bind to a serum protein known as LPS-binding protein (LBP) that transfers an LPS monomer to a glycosylphosphatidylinositol-linked cell surface protein called CD14 the TLR-4 co-receptor. MD-2 is another essential molecule for LPS recognition by TLR-4 (Nguyen et al., 2002). In particular, MD-2 enhances TLR-4dependent NF-kB activity and is needed for LPS-induced TLR-4 signaling (Wright et al., 1990). Following LPS recognition, the TLR-4/MD-2/LPS complex initiates signal transduction by recruiting intracellular adaptor molecules, leading to the activation of NF-κB and mitogen-activated protein (MAP) kinase (Shimazu et al., 1999). This signal cascade induces the transcription of several genes involved in host defense including inflammatory cytokines such as TNF-α, IL-6, IL-1β, chemokines, major histocompatibility complex and co-stimulatory molecules (the TLR signaling pathways are summarized in figure 7).

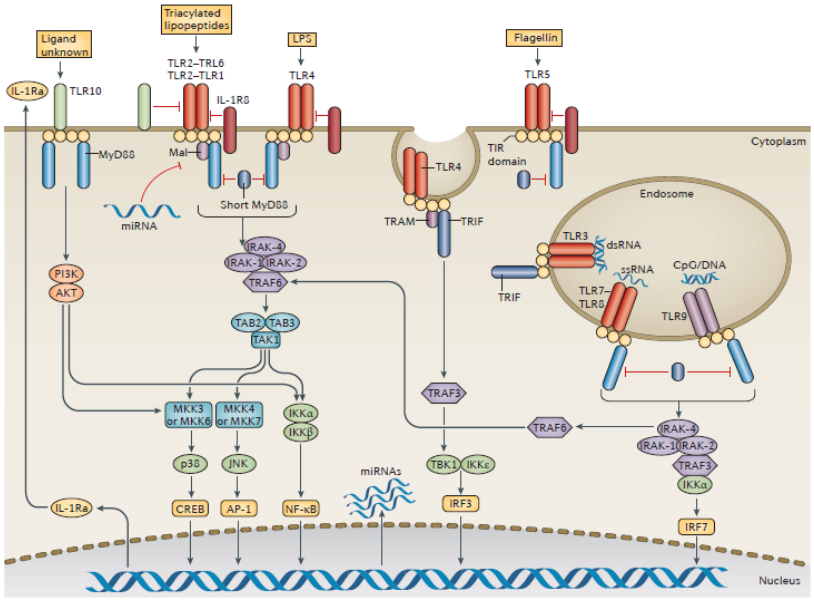


Figure 7. Mammalian TLR signaling pathways (O'Neil et al. 2013). TLR signaling is initiated by ligand-induced dimerization of receptors. Two central adapter proteins MyD88 and TRIF propagate TLR signal transduction by interacting with TLRs via their respective Toll-interleukin (IL)-1 receptor (TIR) components and recruiting down-stream enzymes (e.g. IRAK4, TRAF6) through their 'death' domains. Subsequent modulation of transcriptional control elements is less well defined but occurs through linker molecules such as TAB1, TAK1 and TBK1. Two important families of transcription factors that are activated downstream of TLR signalling are nuclear factor-κB (NF-κB) and the interferon-regulatory factors (IRFs), but other transcription factors, such as cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1), are also important. A major consequence of TLR signalling is the induction of pro-inflammatory cytokines, and in the case of the endosomal TLRs, the induction of type I interferon (IFN). The powerful proinflammatory properties of Gram-negative lipopolysacharide (LPS) may be explained by TLR4-mediated recruitment of both major adapter proteins.

Despite the growing understanding of how LPS mediate toxicity, very few studies have been conducted on microorganisms infecting the respiratory tract of patients with A-T. Cultures established from respiratory secretions performed during acute pulmonary exacerbation identified *Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae, Pseudomonas aeruginosa* and other gram-negative bacilli (Morrow et al., 2008). It remains unclear what the etiology of the lung disease is and how persistent or repeated infections from these microorganisms, together with complications from dysfunctional swallowing and aspiration, contribute to the progress of chronic infections and inflammation of the respiratory traits.

Evidence for the involvement of oxidative stress is accumulating in investigations of patients with A-T, studies in cultured A-T cells and in *Atm*-deficient animal models.

These include elevated levels of reactive oxygen species (ROS) in cells and tissues, protection by antioxidants and the capacity of ATM to recognize and be activated by stimuli that lead to oxidative stress (Kim et al., 2009).

A recent report demonstrated that *S. pneumoniae,* one of the microorganisms identified in the respiratory tract of patients with A-T, caused DNA damage and death of lung epithelial cells by a mechanism that induces oxidative stress (Schroeder et al., 2014). These data suggest that *S. pneumoniae,* together with other microorganisms invading the respiratory tract, may contribute to death of airway epithelial cells and thus to the progressive lung disease in patients.

A separate investigation has shown that loss of ATM impairs inflammasome-dependent anti-bacterial innate immunity as determined by diminished caspase 1 and IL-1β responses after bacterial infection (Erttmann et al., 2016). These authors further demonstrated that oxidative stress was responsible for inhibition of the inflammasome,

impaired innate immunity and higher susceptibility to bacterial infection. This is consistent with increased levels of ROS in A-T cells and in *Atm*-deficient mice.

However, these results appear to be at variance with data from a previous study where the authors revealed that the presence of cytoplasmic DNA enhanced antiviral and antibacterial responses in cells from A-T patients and *Atm*-deficient mice (Hartlova et al. 2015). Contrary to the Erttmann et al. 2016, these results suggest a regular innate immune response in A-T cells and *Atm*-mutant mice.

Overall, the role of oxidative stress and inflammation are emerging as important lines of research to explain A-T pathogenesis. Whether they are interconnected, or represent tissue specific features of A-T remains to a matter of study.

Therapy for A-T.

At present, there is no established treatment that prevents or slows the progression of A-T. Antibiotics and / or periodic infusion of immunoglobulins are used to reduce the risk of recurrent infections, while palliative treatments control neurologic features.

The dietary implement of antioxidant molecules such as vitamin E, vitamin C, N-acetylcysteine and alpha lipoic acid has been proposed based on the rational of an increased ROS stress in A-T cell lines. However, no convincing experimental data have shown a clinical efficacy.

More recently, steroids have been proposed to give a short-term improvement in A-T. Amelioration of neurological signs has been reported after short-term treatment with oral betamethasone (Broccoletti et al., 2011). In a follow-up study, using low doses of betamethasone (0.03 and 0.01 mg/kg/day) with six patients responsive to betamethasone, SARA scores significantly improved in all patients at the higher dose and some improvement was also observed at the lower dose (Broccoletti et al., 2011). These authors suggested that antioxidative mechanisms may have played a role in improving cerebellar functions in A-T (Russo et al., 2009). In a separate randomized trial of 13 patients with A-T, betamethasone reduced ICARS total score significantly (~30%) in both intent-to-treat (all patients randomized) and in per-protocol patients (Zanolli et al., 2012).

Unfortunately, long-term complications of steroid use far outweigh the short-term benefits of this treatment. This led to attempts in the 1980s to focus on the use of these

drugs for long-term treatment of the neurological problems (Gatti et al., 1985). Whilst it was clear that they did reduce symptoms, it seemed equally clear that once discontinued, the symptoms reappeared (Gatti et al., 2009).

Glucocorticoids are known for their anti-inflammatory properties and have been recently used in the treatment of neurometabolic and neurodegenerative pathologies. Although glucocorticoids are highly effective for therapeutic purposes, long-term and/or high dose glucocorticoid administration is associated with adverse side effects, like hyperglycaemia, abdominal obesity, muscle wasting, hypertension, osteoporosis, depression and decreased immunological function. Due to the far reaching and tissue- or cell-specific effects of glucocorticoids on the balance of pro- and anti-apoptotic responses, caution should be implemented in therapeutic strategies to ensure that the great power of glucocorticoids could be utilized minimizing the risks.

To tackle these problems, a system based on intra-erythrocyte delivery of dexamethasone (EryDex) has been proposed. This permits the encapsulation of dexamethasone sodium phosphate to autologous erythrocytes derived from a 50 ml blood sample, processed ex vivo with hypotonic saline which permits osmotic opening of the erythrocyte (RBC) pores and diffusion of dexamethasone phosphate into the cells. Using this approach, a Phase II clinical trial has been recently conducted where patients received a monthly treatment of 50 ml of autologous red blood cells (RBC) loaded with two vials of 250 mg of dexamethasone sodium phosphate. In this study which enrolled 22 patients (18 completed), after six infusions, a mean reduction of 5 points in ICARS was reported. In an extension of this study for an additional 24 months, patients experienced a continuous neurologic improvement with respect to pretreatment status whereas controls showed a progressive neurologic deterioration after discontinuation of the treatment (Leuzzi et al., 2015).

A possible explanation of the molecular mechanisms of dexamethasone on A-T patients, has been proposed. Dexamethasone is reported to induce a non-canonical splicing event of *ATM,* which leads to an alternative splicing variant named ATMdexa1 (mini ATM).

The non-canonical splicing event is mediated by a short direct repeat sequence (SDR) CCTCA which leads to the joining of exon 3 to 52. The resulting cDNA carries the first three exons and the last 11 exons of the native ATM transcript and the lacking of exons from 4 to 51 overpassing all the mutations upstream to this nucleotide (see Fig. 8).

This transcript encodes a protein with a partial restoration of ATM activity on DNA damage response and ATM-dependent cellular signaling (Menotta et al., 2012; Menotta et al., 2017). Moreover, the induction of the ATMdexa1 isoform occurs only in A-T cells, acting as a possible exon skipping therapy in this disease.

To date, dexamethasone has modest neurological effects in a subgroup of patients and it is not a resolutive treatment (Xu et al., 2009).

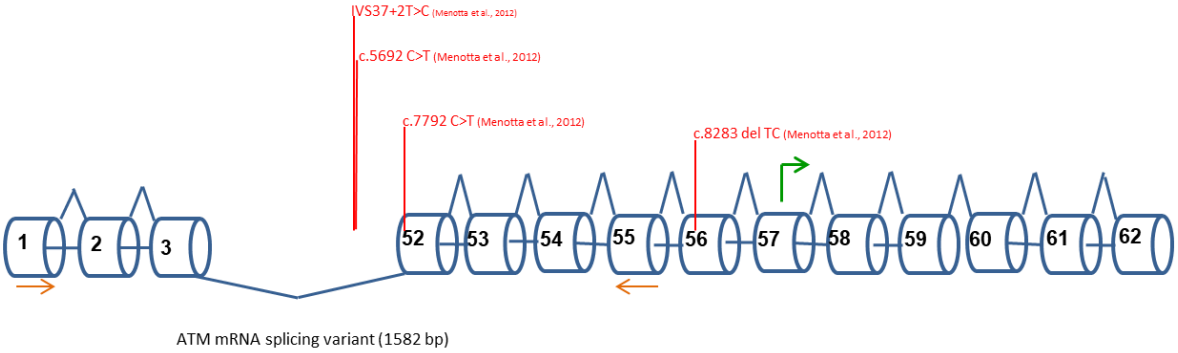


Figure 8. Scheme representing the alternative ATM splicing variant induced by Dexamethasone treatment (Menotta et al., 2012). In red, ATM mutations from A-T LCLs that lead to truncated protein with no detectable ATM levels. The translation of ATMdexa1 escapes the mutations contained in the cDNA maintaining the complete PI3K domain of the native protein. The transcription start site lies in the exon 57 at the position p.2806 (arrow green).

Chapter 2

Aim of the work

In order to gain a better insight on the role of glucocorticoids in the pathogenesis of Ataxia-Telangiectasia, the aims of my work have been:

1) To investigate the *in vitro* effects of glucocorticoid treatment in A-T cell lines in the pathways of DNA damage and oxidative stress.

2) To evaluate the correlation of ATM loss with the defects in the TLRs response pathway. In particular, I focused on TLRs expression and on the correlated chemo-cytokines secretion pattern after NF-kB activation, assuming that A-T patients could exhibit impaired innate immune response due to defective pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs).

Chapter 3
Materials and Methods

Cell isolation, culture and treatment

Two groups were selected for this work:

1) six lymphoblastoid Cell Lines (LCLs) derived from A-T patients carrying the different mutations .Clinical features of these patients are described in results (Tab.1). Four control LCLs, obtained through a collaboration with the Human Genetics Foundation of Torino (HuGeF). Lymphoblastoid cell lines (LCLs), established from AT patients and healthy donors were transformed using the Epstein–Barr Virus (Nilsson et al., 1971) and grown in a RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. Mycoplasma contamination was excluded through a weekly test by PCR using mycolpasma-specific primers. If a line was found to be positive, line was eliminated and a new vial was thawed again. Cell lines were grown until confluence and then stored frozen in fetal calf serum (FCS) supplemented with 10% dimethylsulphoxide (DMSO) at -130°C.

2) six primary skin fibroblasts were obtained from individuals with A-T and healthy controls. Briefly, a forearm skin biopsy was obtained under local anaesthesia and enzymatically digested with 0.25% 0.25% trypsin-EDTA solution (Invitrogen, CA, USA) for 1 hour at 37°C. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/ streptomycin (Gibco BRL) and incubated at 37°C in a 5% CO2 atmosphere. Cells from passages 5 to 9 were used for all experiments and A-T and healthy controls were compared to the same passage. Twenty-four hours after plating, growth medium was changed to DMEM with 5% FBS (and penicillin, streptomycin, and L-glutamine).

For baseline data, cells were cultured without E.coli LPS and TNF-a and for LPS and TNF-a challenge assay, cells were cultured and treated with LPS at 1ug/mL from 3 hrs to 48 hrs and TNF-a 20ng/mL from 3hrs to 24 hrs.

For Dexamethasone treatment, cells were cultured for 72 hrs at a concentration of 0.1uM of dexa. Cells were irradiated at 10 Gy, and after 1 hrs were lysate and collected for immunoblot analysis.

Total RNA extraction and cDNA generation.

Total RNA was extracted from $5x10^6$ LCLs and from $2X10^5$ fibroblasts using the Direct-zol™ Kits- RNA extraction kit (Zhymo research) according to manufacturer's instructions. One

microgram of RNA was retrotranscribed using the M-MLV Reverse Transcriptase following manufacturer's conditions (Thermo Fisher Scientific).

Primer sequences designed to amplify the entire ATM transcript were:

forward 5'-AGTCTAGTACTTAATGATCTGCTTATCTGC and reverse 5'-CCACCTTATAAGTACAGATAGTTAATTTCC. Suitable primers, in the exons 26 and 27 to detect the native ATM and surrounding the noncanonical spliced ATMdexa1 junction (in exons 2 and 52), were designed following data from (Menotta et al., 2012):

- Native ATM: 5'-AAATTCTAGTGCCAGTCAGAGC, 5'-TTGCTTTAATCACATGCGATGG
- ATMdexa1: 5'-ATCTAGATCGGCATTCAGATTCCA , 5'- GTTTAGTAATTGGCTGGTCTGC

The specificity of the amplification products was confirmed by examining thermal denaturation plots and one peak is detectable by melting curve analysis, confirming that the assay condition was optimal.

Cycle conditions were 95 °C for 10 min followed by 40 two-step cycles of 20 s at 94 °C and 40 s at 70 °C for B-actin and ATM full length.

Amplification plots were analysed using the 7500 System. The specificity of the amplification products was confirmed by examining thermal denaturation plots, by sample separation in a 1% DNA-agarose gel.

Validation of data by real time RT-PCR

Total RNA was extracted from LCLs and fibroblast cell lines with the Direct-zol™ Kits- RNA extraction kit (Zymo research) from an A-T patients and healthy controls. One microgram of total RNA was retrotranscribed as described above.

Quantitative real-time RT-PCRs were carried out on an ABI-Prism7500 Fast instrument (Applied Biosystems) using the Taqman Gene Expression Master Mix (Applied Biosystems), Universal Probe Library (UPL) technology (Roche) and according to the manufacturer's protocol. Primers and UPL probes used for real time validation are shown in table 4.

Table 4. Primer list for qRT-PCR.

Gene	Locus	Forward primer 5'-->3'	Reverse primer 5'-->3'	UPL probe
TLR4	NM_138554.4	cctgcgtgagaccagaaag	ttcagctccatgcattgataa	10
IL6	NM_000600.4	gatgagtacaaaagtcctgatcca	ctgcagccactggttctgt	40
CXCL8	NM_000584.3	agacagcagagcacacaagc	gcactccttggcaaaactg	72
CAT	NM_001752	gctcattttgaccgagagaga	tgacctcaaagtagccaaagg	68
GPXI	NM_002083	caaccagtttgggcatcag	tctcgaagagcatgaagttgg	77
SOD1	NM_000454	tcatcaatttcgagcagaagg	gcaggccttcagtcagtcc	60

UPL technology is based on 165 short hydrolysis probes (8-9 nucleotides-long), labelled at the 5' end with fluorescein (FAM) and at the 3' end with a dark quencher dye, that hybridize the human transcriptome: each probe binds to approximately 7000 transcripts, while each transcript is detected by approximately 16 different probes. To maintain the specificity and melting temperature (Tm) of the probe, Locked Nucleic Acids (LNAs) are incorporated into the sequence of each UPL probe. LNAs are DNA nucleotide analogues with increased binding strengths compared to standard DNA nucleotides. Only one specific transcript is detected at a time in a given PCR assay: the specificity is given by the set of chosen PCR primers.

Experimental Ct values were normalized to TATA-binding protein, used as endogenous control (VIC labelled pre-designed TaqMan gene expression assays, TBP, Hs00427620_m1, Applied Biosystems). Gene expression was calculated in each sample relative to the mean of controls, using the formula 2-ddCt, where dCt is Ctgene-Ctendo and ddCt is dCtsample- mean dCtcontrols as described in (Livak and Schmittgen, 2001). Each sample was examined in triplicate and differences in gene expression of patients relative to controls were statistically evaluated by *t* test for at least three independent experiments.

Analysis of CAT activity

The day of the experiment, a total of 1×10^6 cells, obtained from five A-T cell lines and three control cells, were collected by centrifugation, washed twice in PBS and homogenized in ice-cold lysis buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.0). Cells were centrifuged at 10,000g for 15 min at 4 °C. Supernatant was stored on ice for

the assay. To evaluate CAT activities, we used the Catalase Assay kit (Cayman, MI, USA, #707002) following manufacture's protocol. Analysis was performed by monitoring the absorbance at 540 nm on an xMark microplate Reader (Bio-Rad Laboratories S.r.l., Segrate, Italy). CAT activity was defined as the amount of CAT enzyme able to produce 1.0 nmol of formaldehyde per minute at 25 °C, interpolating the values in a standard curve of formaldehyde ranging 0–75 µM. Each sample was assayed in duplicate and in at least three independent experiments.

DCFH-DA assay

Intracellular Reactive Oxygen Species (ROS) levels were determined by using a fluorescent dye, DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) (5 µM). DCFH reacts with ROS to generate a new highly fluorescent compound, dichlorofluorescein, which can be analysed with flow cytometry (FACS). Treated cells were incubated by DCFH-DA (5 µM) at 37°C for 30 min, washed twice with PBS, and then measured with FACS Calibur flow cytometer using Cell Quest software (Becton Dickinson, USA). Cells were treated with Dexa 0,1uM for 72 hours and with TBH (300 µM) that is a stable chemical form of H_2O_2 for 30 minutes as a positive control of the experiment.

Three independent experiments were done for each method.

Western Blot

Total Lysate Proteins were extracted from LCLs or fibroblast cell lines of six A-T patients and four healthy controls, using RIPA Lysis Buffer [TrisHCl (50 mM), NaCl (150 mM), Triton (1%)] and quantified using standard Bradford protocol (Fermentas). Nuclear lysate was extracted from fibroblast cell lines using (Thermo Scientific) NE-PER Nuclear and Cytoplasmic Extraction Kit and quantified as described above. Twenty ug of proteins were denaturated for 10 min at 70°C in Laemli Buffer and Reducing Agent (Thermo Scientific) and separated by SDS–PAGE (4-12%), using a Tris-Glycine-SDS Buffer. Transfer was performed on nitrocellulose membranes (Biorad) using a wet apparatus (Biorad) and a Tris-Glycine Buffer added with 20% methanol, for one hour and half at 125 V. 5% milk (Biorad) in TBS-T 0,1% (Invitrogen) was used for blocking, and 1% milk for hybridization of antibodies and their detection was performed, following manufacturer's instructions. Hybridizations were performed with primary antibodies against pATM(1:5000),

pH2A_X(1:3000), p65_NF-kB (1:1000), pAKT (1:2000) (Abcam). Beta-actin (1:2000) antibody or Vinculin (1:2000)(Abcam) antibody were used as loading controls.

For all western blot analysis, we synchronized cells with an overnight incubation in RPMI or DMEM medium supplemented with 5% FCS. Cells were then grown for 72 hours in RPMI or DMEM medium supplemented with 5% FCS, before lysis for total or nuclear proteins preparation.

ELISA assay (Immunoassays of IL-6)

The amounts of secreted cytokines in culture supernatants of fibroblast cell lines of five A-T cells and five healthy controls were measured by sandwich ELISA. Briefly, media containing 4 μg/ml monoclonal antibodies to each cytokine were placed in 96-well culture plates and incubated overnight at 4°C. The next morning, the plates were treated with the blocking solution (1% BSA and 0.05% Tween 20 in PBS) for 2 hours at room temperature, the supernatants to be tested and standard recombinant cytokines were added to each well, and incubation was continued. After 2 hours, 500 ng/ml of biotinylated monoclonal antibodies to each cytokine was added and the reactions were allowed to proceed for another 2 hours at room temperature. Next, streptavidin-conjugated alkaline phosphate (Sigma) was added to make a 1 : 2000 dilution, and cells were incubated again for 2 hours at room temperature. Finally, a colour reaction was induced by adding 1 mg/ml of *p*-nitrophenylphosphate (Sigma) dissolved in diethanolamine (Sigma) and was stopped by adding 1 N NaOH. Every time new reagents were added to the well, the plates were washed 4 times with PBS containing 0.1% Tween 20. The optical density of color reactions was measured with a Vmax automated microplate reader (Molecular Devices, Palo Alto, CA, USA) set at 405 nm. Standard curves were drawn by plotting optical density versus the concentration of each recombinant cytokine in a logarithmic scale.

FACs analysis

Flow Cytometry for TLR-2 Expression on fibroblast cell lines. Briefly, 50 μL of fibroblasts cells were incubated with the following primary antibodies for 30 minutes on ice: PE-conjugated anti-TLR2 (1 μg, clone TL2.1, eBioscience, San Diego, CA. Isotype-matched irrelevant antibody controls were used to detect nonspecific staining. The stained cells

were washed three times with wash solution (FACS; BD Biosciences) and the cells fixed with 1% paraformaldehyde.

Flow cytometer (FACScan; BD Biosciences) was used for data acquisition. Ten thousand events were acquired for the fibroblasts cellular gates. Analysis of the acquired flow cytometry data was then performed (CellQuest software, ver. 3.1 for Macintosh; BD Bioscience Immunocytometry Systems, San Jose, CA). Mean channel fluorescence intensity (MFI) derived from fluorescence histogram was used to study the level of cell surface TLR expression. MFI was calculated as a ratio and recorded as the MFI of the TLR2 divided by the MFI of the isotype-matched negative control antibody. Therefore, an MFI ratio of 1 represents no significant expression of the TLR under investigation, and an MFI ratio >1 represents higher levels of expression.

TUNEL assay

TUNEL is a method for detecting apoptotic DNA fragmentation, widely used to identify and quantify apoptotic cells, or to detect excessive DNA breakage in individual cells.

DNA fragmentation was assessed in situ using a fluorescein-based In situ cell death detection kit (Roche, Penzberg, Bayern, Germany) as described by the manufacturer, with minor modifications. Fixed fibroblast cell lines of one A-T and one healthy control were placed in 0.01 M phosphate buffered saline (PBS) for 10 min, and treated with 0.03% Triton X-100 for at least 15 min at room temperature. After washing twice with deionized water for 2 min, each culture was immersed in 1× TdT labeling buffer for 5 min. The cultures were incubated in TUNEL mix containing 50× TdT dNTP, 50× cation (Mg^{2+}), 50× TdT enzyme, and 1× TdT labeling buffer for 60 min at 37°C in a humidity chamber. The reaction was terminated by washing in 1× TdT stop buffer for 5 min. The cultures were washed in 0.05% PBS-Tween 20 (PBS-T), before being mounted on glass with coverslips using Tris buffer containing flourogel (Electron Microscopy Sciences). Samples were viewed using a fluorescence microscope (Nikon Eclipse 80i-Vico) using a 495 nm filter.

The images are enlarged to 40x. Magnification bars are 50 µm.

Generation of ATM knockout cell line by using CRISPR/Cas9 method

HeLa CRISPR/ATM KO cells were produced by Dr Maria Vinciguerra (IFOM, DNA Metabolism Unit, Milano, Italy). An online CRISPR Design Tool (http://tools.genome-engineering.org) was used in order to select the genomic sequence of interest, identify suitable target sites (taking into account the 5'-NGG PAM for S. pyogenes Cas9 and the minimization of off-target activity) and design the sequences for oligos and primers necessary to prepare the sgRNA constructs. Complementary oligonucleotides were annealed, phosphorylated and cloned into the lentiCRISPR plasmid (Addgene 49535), expressing human codon-optimized Cas9 protein and puromycin resistance from EFS promoter and CRISPR chimeric RNA element with customizable sgRNA from U6 promoter.

To make lentivirus, the LENTICRISPR plasmid was co-transfected into HEK293-T cells together with the packaging plasmids pCMV-VSV-G (AddGene 8454) and psPAX2 (AddGene12260). As a positive control for viral production, was used a pLJM1--EGFP lentiviral transfer plasmid (eg. AddGene19319).

Respectively 24 and 48 hours later, 293-T medium, containing the lentiviral particles produced, was used to infect HeLa cells. Two days after the infection, cells were selected by treatment with puromycin for three days. Single cell dilution and clonal expansion followed. After two weeks, only green clones (co-expressing GFP) were screened by T7 endonuclease (T7E1) assay and Western blot, in order to check the activity of cas9, the specificity of the cut and the final effect on ATM protein expression.

Statistical analysis

Statistical analysis was performed using Graphpad software (www.graphpad.com) .

Chapter 4
Results

1. Evaluation of dexamethasone treatment in A-T cell lines.

1.1: Analysis of ATM transcript variants induced by dexamethasone.

Dexamethasone treatment of A-T cell lines is reported to induce the formation of an alternative functional transcript of the *ATM* gene (ATMdexa1) (Menotta et al 2012).

To validate this finding, we collected six lymphoblastoid cell lines (LCLs) derived from A-T patients (AT-18; AT-34; AT-36; AT-38; AT-90; AT-0205) with different gene mutations as reported in table 4, and two healthy controls.

The mutations in our patients hit exons outside the ATMdexa1 encoding region on one or both alleles with one exception: AT-38 carrying two nonsense mutations in exons 63 and 65 (Fig. 9).

We initially estimated a dose curve for dexamethasone and found that >1 µM for 24 hours induced cell toxicity in LCLs using MTT assay. Thus, we treated cell lines with two 10-fold difference concentration of dexamethasone (0.1 µM and 1 µM) for 24 hours. After total RNA extraction, cDNA was retrotranscribed and amplified using a sense primer on exon 1 and an antisense primer on exon 55 of the *ATM* cDNA following data from (Menotta et al., 2012).

Table 4. A-T patients analysed in this study.

ID	Genotype	ex/int	Mutation	Consequence	ATM levels	Kinase activity after Gy	note	human cell lines
AT-2	Compd Htz	6	c.331A>T	p.Arg111*	absent	no ATMp	mild phenotype	Fibroblasts
		65	c.9104C>T	p.Leu3035Phe				
AT-18	Hom	53	c.7517_7520delGAGA	p.(Arg2506fs); p.(Arg2506fs)	absent	no ATMp	classic A-T	LCLs
AT-34	Compd Htz	45	c.6326G>A	p.(Trp2109*)	less than 10%	no ATMp	mild phenotype	LCLs
		IVS11	c.1236-405C>T	p.(Trp412*)				Fibroblasts
AT-36	Compd Htz	23; IVS55	c.3111delT; c.7928-1G>A	p.[(Ser1037fs*); Lys2643_Lys2671del)]	absent	no ATMp	classic A-T	LCLs
		38	c.5441T>A	p.(Trp1814*)				
AT-38	Compd Htz	63	c.8814_8824del11	p.(Met2938fs*)	absent	no ATMp	classic A-T	LCLs
		65	c.9169T>G	p.(*3056Glyext*28)				
AT-39	Compd Htz	4	c.-30_2816dup41Kb	exons 4-20 duplicated	absent	no ATMp	classic A-T	Fibroblasts
		IVS6	c.331+2T>G	Ab splice (del ex 6)				
AT-46	Compd Htz	12	c.1369C>T	p.(Arg457X)	absent	no ATMp	classic A-T	Fibroblasts
		26	c.3576G>A	p.(Ser1135_Lys1192del58)				
AT-90	Compd Htz	26	c.3576G>A	p.(Ser1135_Lys1192del58)	15%	25-30%	late onset	LCLs
		35	c.4910-3T>A	p.(Asp1637_Val1654del17)				
AT-0205	Compd Htz	26	c.3576G>A	p.(Ser1135_Lys1192del58)	less than 10%	no ATMp	classic A-T	LCLs
		IVS12	IVS12+1G>T	p.(Cys536fs*)				Fibroblasts
AT-8492	Compd Htz	16	c.2250G>A	p.(Glu709_Lys750del42)	absent	no ATMp	mild phenotype	Fibroblasts
		57	c.8122G>A	p.(Asp2708Gln)				

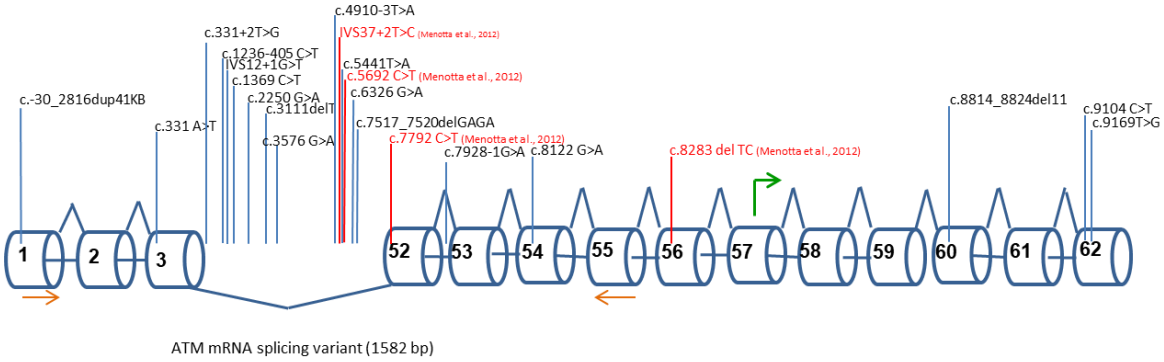
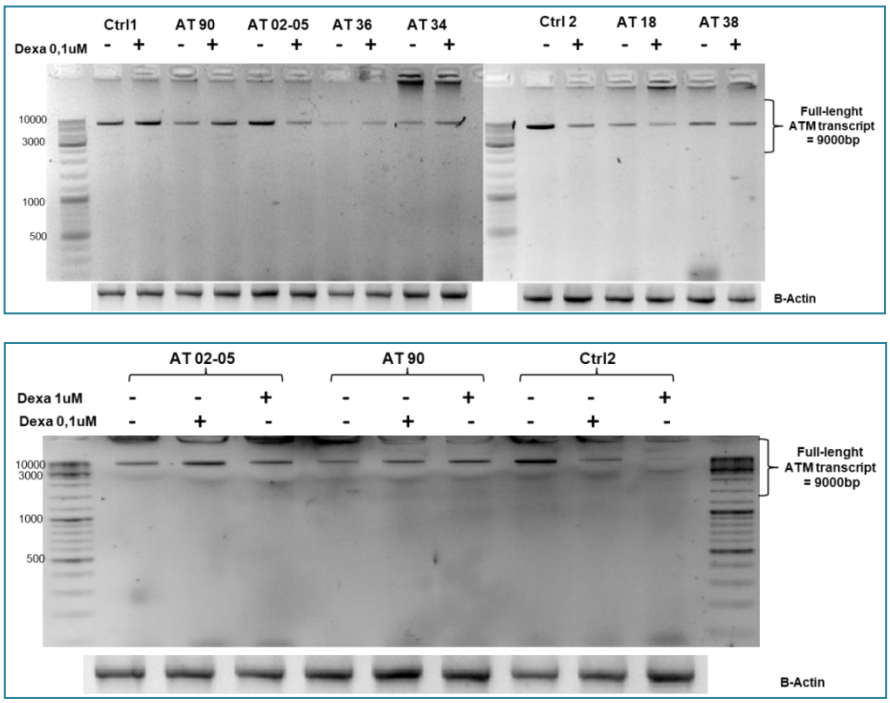


Figure 9. Schematic representation of the alternative ATM splicing variant induced by dexamethasone (Menotta et al., 2012).
In red, position of the mutations from A-T LCLs described by Menotta et al. 2012; in black, positions of the mutations from our LCLs and fibroblast cell lines.

In none of our patients' cell lines, we detected the expected 1,600 bp band, corresponding to the ATMdexa1 transcript. To verify our method did not fail to amplify the transcripts, we were able to see a residual amount of "full-length" *ATM* (~9 kb) escaping nonsense mediated decay (Fig. 10). Even if in some cases a faint increase of *ATM* was seen after dexamethasone, densitometry quantification of the bands did not show any statistical difference of expression between treated and untreated cell lines.

To verify the activity of dexamethasone, we measured IL-8 expression as read-out on fibroblasts. As expected, treated cells showed a reduction of IL-8 gene expression (see paragraph 6; fig. 29-30) (Hermoso et al., 2004; Mogensen et al., 2008).



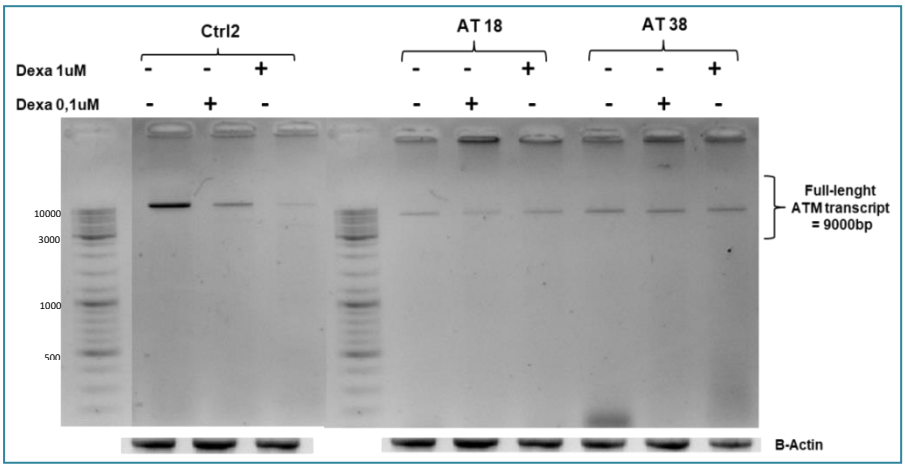


Figure 10. Dexamethasone treatment of A-T LCLs.
The full-length ATM transcript was amplified by RT-PCR on total cDNA from A-T and control LCLs treated or not with dexa 0.1 µM and 1 µM. No further band could be evidenced.

To validate our results, the expression of native *ATM* and ATMdexa1 transcripts were evaluated by quantitative SYBR-green real-time PCR, with or without dexamethasone treatment, in both fibroblasts and lymphoblastoid cell lines.

Native *ATM* was detected in all A-T and controls, without significant changes following dexamethasone treatment (Fig. 11). In some A-T cell lines the expression of *ATM* was only mildly reduced, mostly due to the type of mutation(s). For instance, AT-90 showed a level comparable to controls. This case has a late-onset phenotype and two splicing mutations.

Conversely, the expression of the ATMdexa1 transcript was undetectable in all cell lines both from A-T and controls (Fig. 12). Also the analysis of the melting curve profile did not support the presence of a transcript.

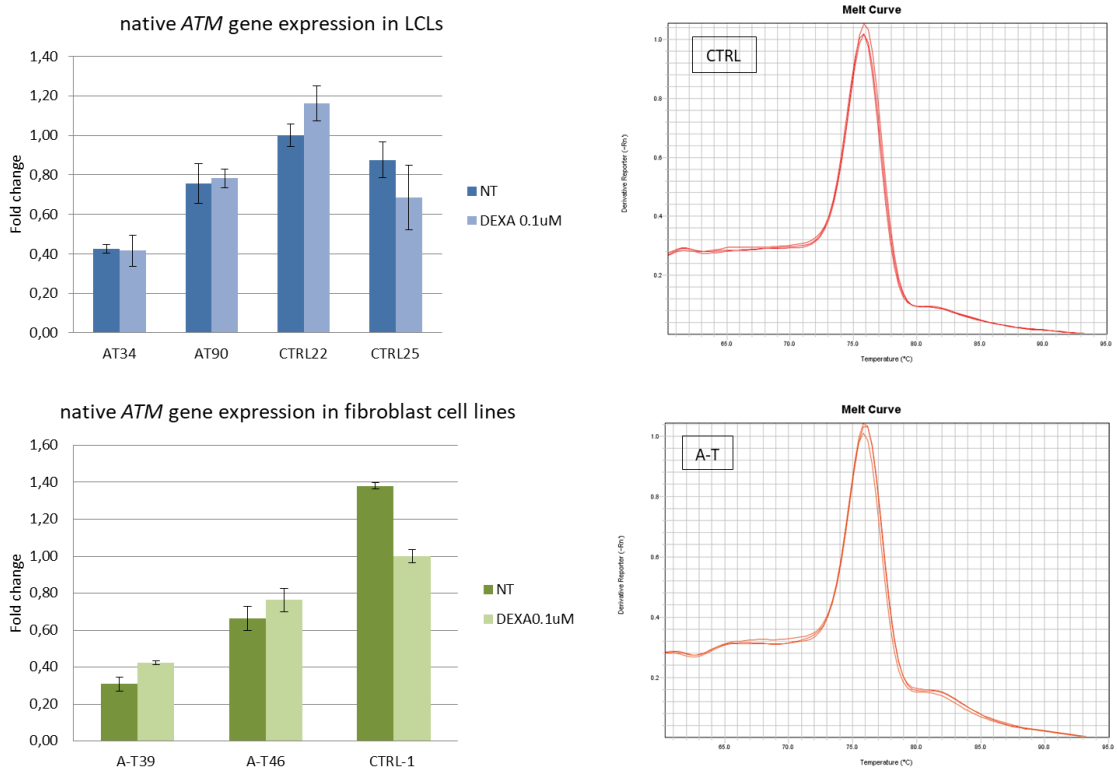


Figure 11. Quantification of full-length *ATM* by qRT-PCR. Full-length ATM transcript was quantified in A-T34 and AT-90 and two healthy controls LCLs and in AT-39, AT-46 and one fibroblast cell lines with or without dexamethasone treatment for 24 hrs. No difference could be detected after dexamethasone stimulation.

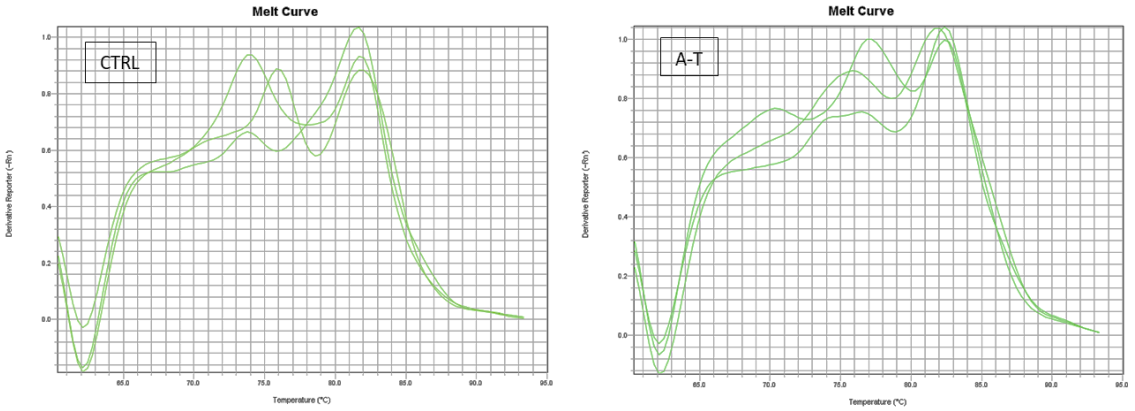


Figure 12. Quantification of ATMdexa1 by qRT-PCR. ATMdexa1 transcript was quantified in AT-34 and AT-90 and two healthy controls LCLs and in AT-39, AT-46 and one fibroblast cell lines with or without dexamethasone treatment for 24 hrs. Example of both conditions: nonspecific peak is detectable by melting curve analysis supporting the formation of one transcript that is not specific in both cell lines.

2. ATM-dependent cellular signaling pathway analysis.

2.1: Nuclear ATM: H2AX phosphorylation (γH2AX).

Because dexamethasone was also reported to induce ATM-dependent responses, we further analysed these data reported by Menotta et al. (2012).

Initially, we analysed the activation of γH2AX, a well-known ATM substrate, and an early player in DNA double strand break (DSBs) repair response. Phosphorylation of H2AX by ATM is lost in A-T cell lines, while ATMdexa1 is suggested to regain a kinase activity on H2AX after DSB induction.

Using nuclear protein extracts from CTRLs and A-T cell lines, we analyzed the activation pattern of our LCLs exposed to dexamethasone 0.1 µM. In particular, we tested one "classic" (AT-18), one "mild" (AT-34) and one "late-onset" A-T case (AT-90). In both controls and A-T LCLs, γH2AX was present without any treatment, suggesting a basal activation of this protein even in the complete absence of ATM.

After dexamethasone treatment, we showed a 30-40% increase of H2AX phosphorylation (Fig. 13). A similar result was obtained after DSB induction by ionizing radiation (Fig. 14). This increase was clearly visible in A-T cell lines where H2AX phosphorylation was even higher in cells treated with dexamethasone after irradiation, suggesting a direct role of the drug treatment in improving the DSBs response, in all cell lines, with some variability.

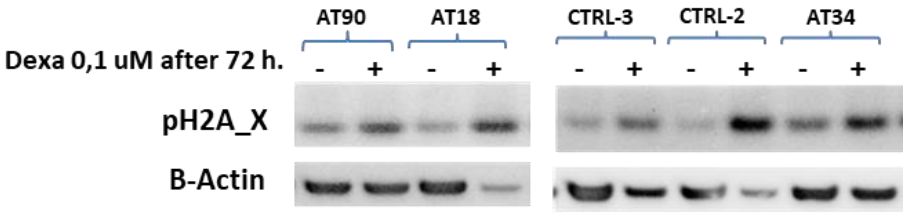


Figure 13. DNA damage response activation after dexa treatment in A-T cells was assessed by H2AX phosphorylation in Ser-139, which is a direct substrate of ATM. The densitometry analysis performed and data presented as Mean ± SE of three independent experiments (*n* = 3).

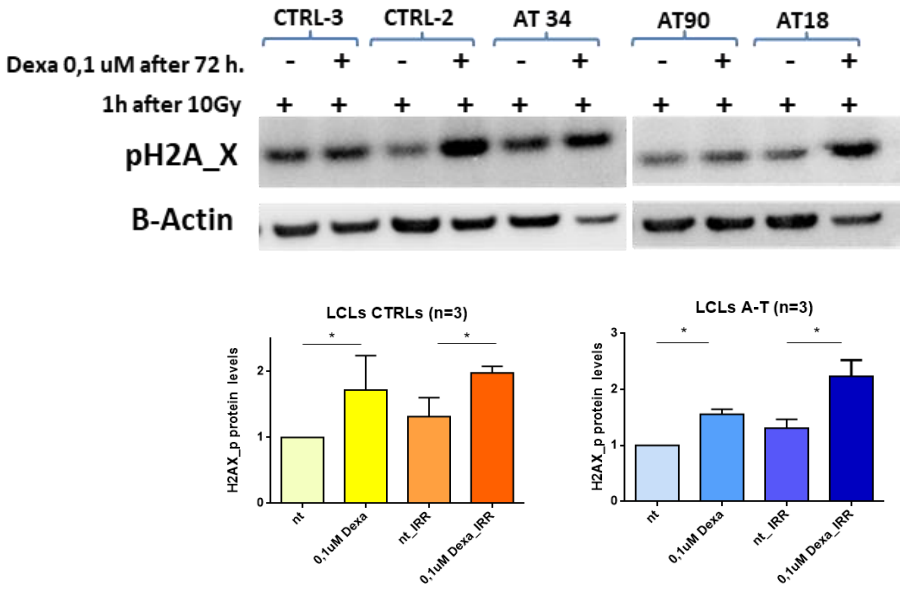


Figure 14. DSB induction in A-T cell lines.
γH2AX increased after dexa treatment both in A-T and control cell lines. The densitometry analysis performed and data presented as Mean ± SE of three independent experiments (*n* = 3).

Because DNA-PK acts redundantly with ATM in DNA damage response, we tested if this protein could be responsible for both basal and DSB-induced activation of γH2AX. After 72 hours of dexamethasone treatment, A-T and control LCLs were challenged with NU7441, a specific inhibitor of DNA-PK, for 1 hour, and then irradiated in order to induce DSBs. We showed that inhibition of DNA-PK activity decreased H2AX phosphorylation levels after dexamethasone treatment, confirming that the effect on γH2AX attributed to the ATMdexa1 could depend on DNA-PK (Fig. 15).

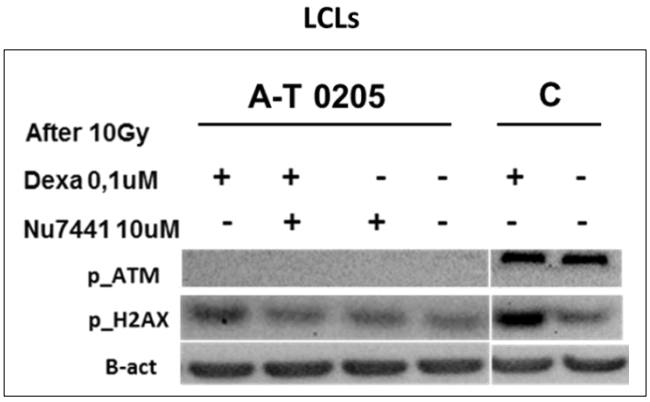


Figure 15. DNA-PK role in γH2AX activation.
In A-T LCLs, we assessed H2AX phosphorylation in Ser-139, which is a direct substrate of ATM but also of DNA-PK.

Given that LCLs are transformed cell lines and showed high basal γH2AX levels, we performed the above experiments also on primary A-T skin fibroblasts (AT-34, AT46), showing that γH2AX basal levels were lower than in LCLs (Fig. 16). After irradiation, γH2AX was enhanced but its phosphorylation was clearly inhibited after treatment with NU7441 DNA-PK inhibitor, which selectively acted on A-T cells and not in control fibroblasts, having these a functional ATM protein. However, in contrast to LCLs, the increase of γH2AX levels after dexamethasone stimulation was very weak and not statistically relevant, both in A-T and control fibroblasts, suggesting that dexamethasone treatment is not able to increase H2AX phosphorylation levels.

Overall, our data show the activation of H2AX in A-T fibroblasts is DNA-PK-dependent, and that the use of LCLs must be avoided due to cell-specific artifacts.

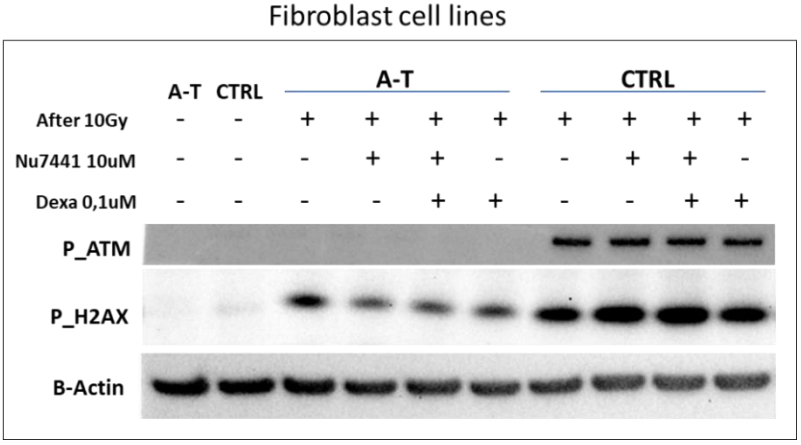


Figure 16. A-T fibroblast analysis of DNA-PK. In figure, AT-34 fibroblast cell line is reported as an example. DNA-PK action was assessed by H2AX phosphorylation in Ser-139, which is a direct substrate of ATM and DNA-PK.

2.2: Cytoplasmic activity of ATM: AKT phosphorylation.

Following experiments on γH2AX, we decided to evaluate other downstream effectors of ATM. It is known ATM stimulates insulin induced AKT phosphorylation at Ser473 and mediates the full activation of AKT activity (Halaby et al., 2008; Yang et al., 2011). AKT participates in multiple physiological processes, including protein translation, glucose uptake, cell proliferation and cell survival, in response to many growth factors.

AKT1 is catalytically inactive in serum-starved primary and immortalized fibroblasts, while in our set up experiment, we showed control cell lines had a strong activation of p-AKT. This activation was reduced in a dose-dependent manner at reducing the serum percentage (Fig. 17).

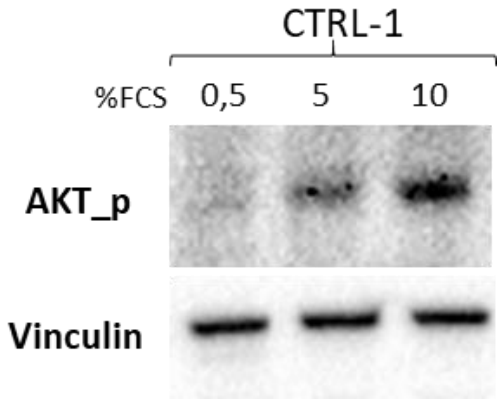


Figure 17. AKT activation in one control fibroblast cell incubated with 0.5%, 5%, and 10% FBS.
Serum is capable of activating AKT in healthy control cells.

Interestingly, the A-T cell lines had low level of p-AKT even at high serum levels, demonstrating the phosphorylation of AKT, upon extracellular stimuli, is ATM dependent (Fig. 18). Indeed, this activation relies on the cytoplasmic ATM, and not on its nuclear function, which is inactive in the absence of DSB.

At high serum level, irradiation in controls showed a progressive reduction of p-AKT, which, on the other hand remained unchanged in A-T.

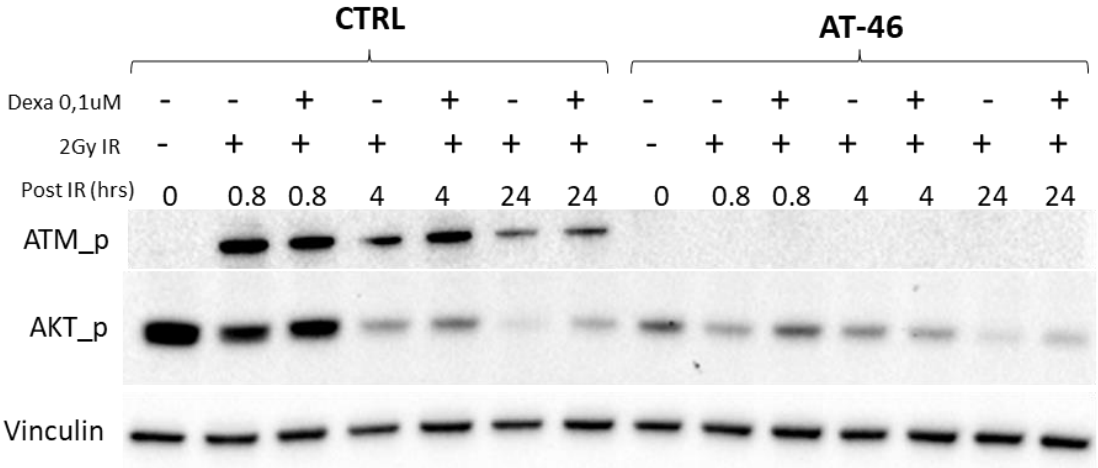


Figure 18. The time course of AKT activation following 2 Gy of radiation in one A-T and one control cells. Cells were incubated in 10% FBS containing medium. Total cell lysate was prepared at 0, 0.8, 4, 24 hours following radiation.

Thus, to explore the effect of IR on AKT, we used a low-serum A-T and control fibroblast cell lines that were treated with dexamethasone 0.1 µM for 72 hrs and/or irradiated with low dose gamma ray (2 Gy). Cell lines showed an increase of pAKT levels both at basal conditions and after ionizing radiation (Fig. 19). This increment was higher in controls compared to A-T cells, likely because normal cells have both ATM and DNA-PK that synergistically act on AKT.

Overall, our data show dexamethasone mainly activates AKT through ATM, although a modest activation takes place also through other PI3Ks.

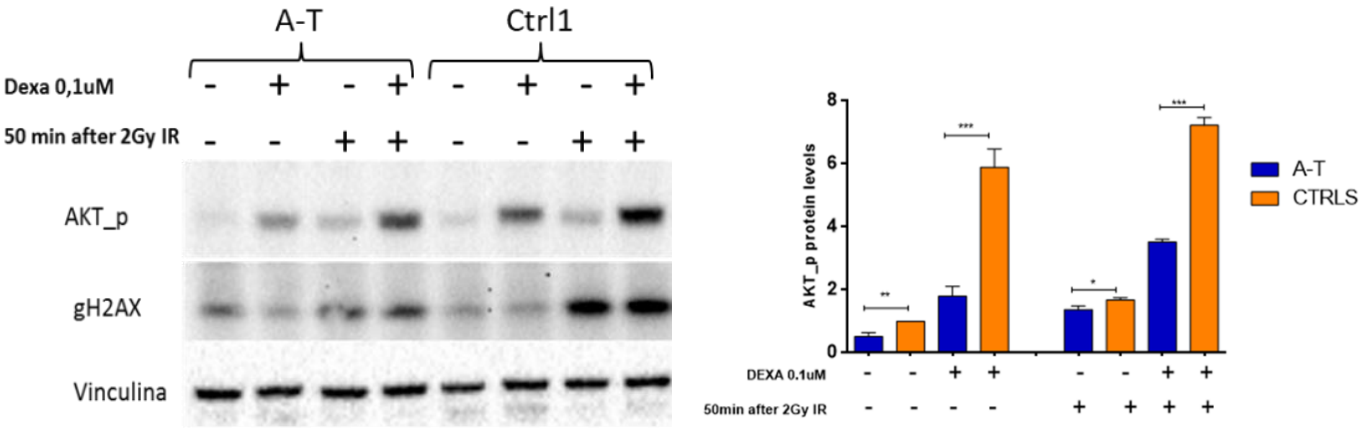


Figure 19. Analysis of AKT in fibroblasts from A-T patients. The figure reports the example of AT-46 (A-T). Histograms refer to cases AT-46 and AT-34. Cells were incubated in 5% FBS containing medium.The densitometry analysis performed and data presented as Mean ± SE of three independent experiments (*n* = 3).

3. Reactive Oxygen Species (ROS) response.

In agreement with the literature (Watters et al., 2003; Pallardó et al., 2010; Valentin-Vega et al., 2012; Perrone et al., 2016), we showed that A-T fibroblast cell lines had a constitutive higher level of intracellular ROS compared to healthy controls, using the DCFH-DA assay (Tarpey et al., 2004) (Fig. 20, Squadrone et al., 2015). After72 hrs of dexamethasone treatment, we found significantly increased ROS levels (~30%, figure 20A). This increase was detected both in A-T (n= 5) and control cell lines (Fig. 20B).

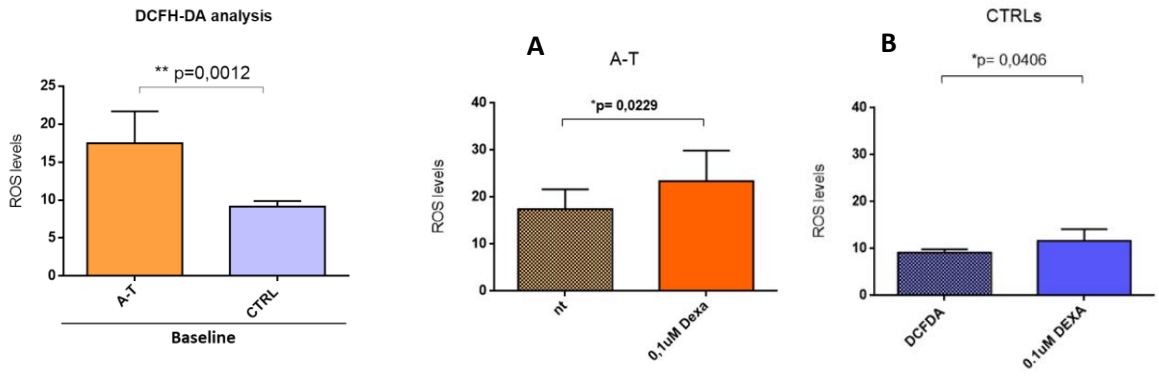


Figure 20. ROS levels in control and A-T fibroblast cells.
ROS levels, measured using a DFCH-DA assay, are increased in A-T cells compared to controls.

To test whether dexamethasone anti-inflammatory action cooperates with the anti-oxidant defense system, we investigated gene expression levels of oxidative stress enzymes CAT, GPX1, SOD1 and SOD2 in A-T fibroblast cell lines and healthy controls both at basal level and after dexamethasone treatment.

At basal level, in A-T fibroblasts cell lines (n=5) catalase (*CAT*) and Glutathione Peroxidase (*GPX1*) gene expression were significantly increased compared to controls (Fig. 21) while the expression of Zn-SOD (*SOD1*, fig. 21) and Mn-SOD (*SOD2*, data not shown) enzymes did not change. After dexamethasone treatment, *GPX1* gene expression decreased (Fig. 21).

Taken together, our results show that A-T cell lines have augmented ROS levels, which further increase when challenged with dexamethasone, while some detoxifying enzyme as GPX1 is reduced.

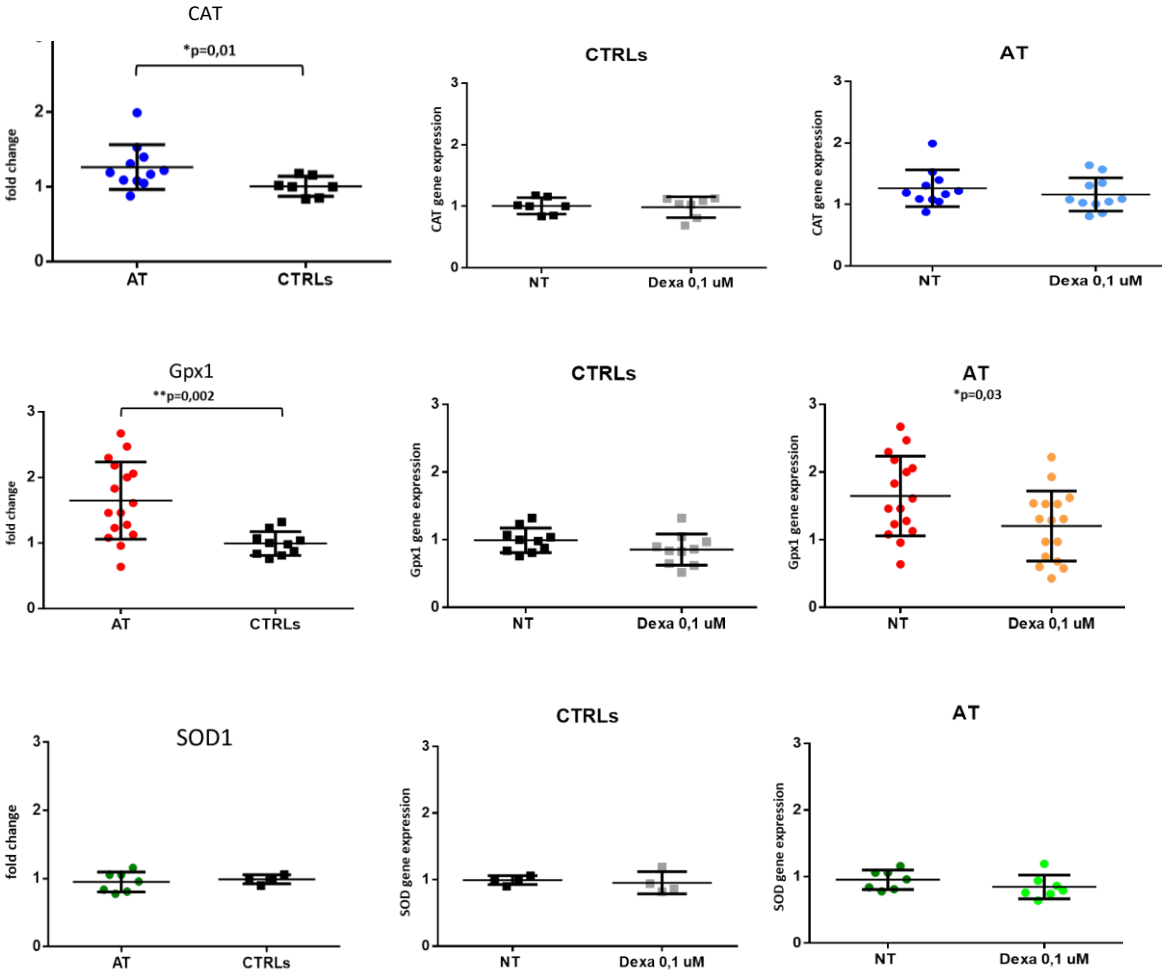


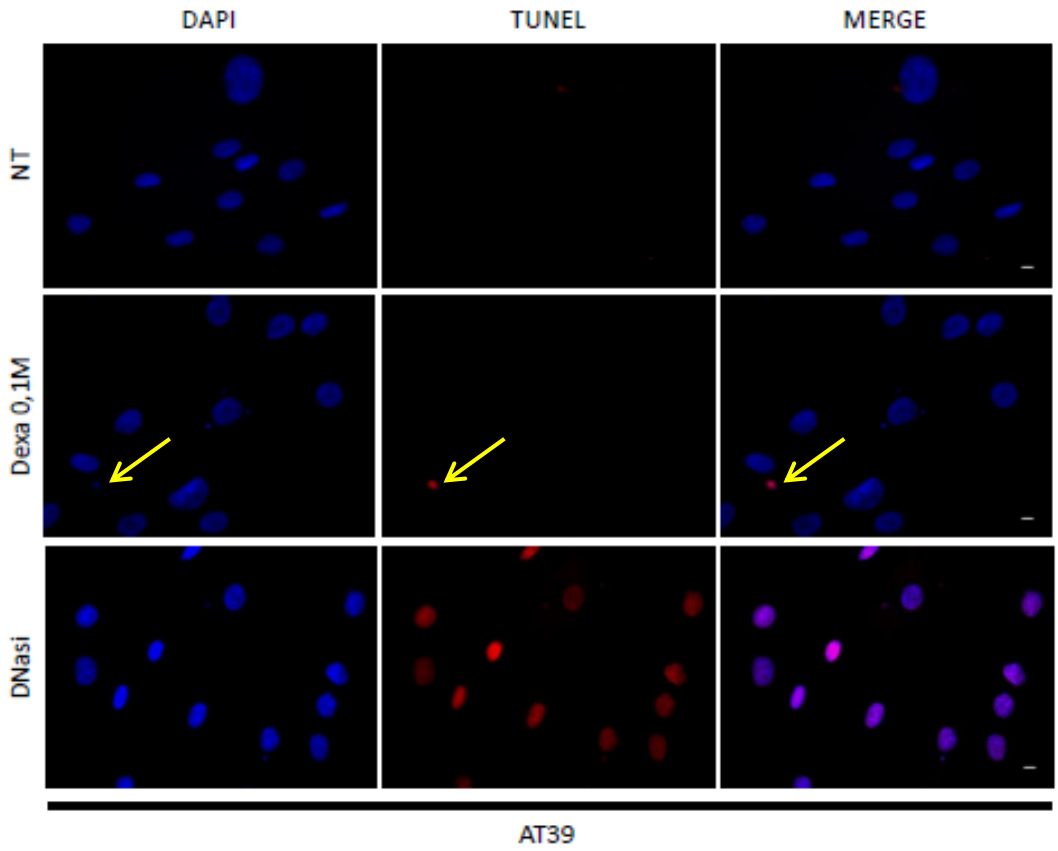
Figure 21. *CAT, GPX1,* and *SOD1* gene expression measured by qRT-PCR in A-T and control fibroblasts.

4. Assay of cell-death following dexamethasone treatment.

To verify this potential deleterious effect of dexamethasone, we investigated its role on promoting cell apoptosis by increased ROS. We evaluated programmed cell death by quantifying TUNEL assay and DAPI-stained condensed nuclei on one fibroblast A-T cell line (AT-39) and one control, after 72 hrs of dexamethasone treatment. We found ~2% of TUNEL-positive cells in A-T vs. 0.5% in control (Table 5, fig. 22), a non-statistically significant difference, indicating that treatment with dexamethasone may affect mildly apoptosis (Fisher Test: p-value > 0.05) (Fig. 22).

	AT39			CTRL		
	Tunel positive	Dividing cells	Total nuclei	Tunel positive	Dividing cells	Total nuclei
NT(C-)	0	7	43	0	12	48
DEXA 0.1uM	5	14	225	1	60	211
Dnasi (C+)	13	/	13	11	/	11

Table 5. Number of TUNEL-positive cells relative to DAPI-positive total nuclei.


DAPI
TUNEL
MERGE
NT
Dexa 0,1M
DNasi
AT39

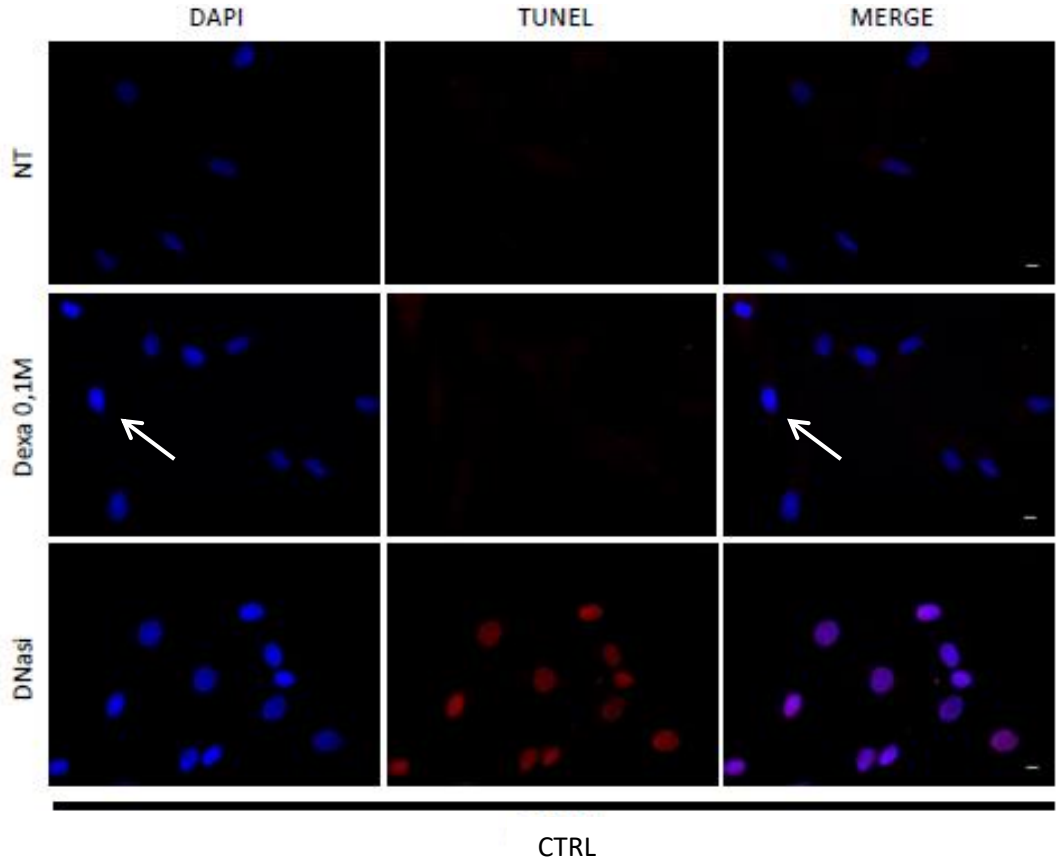

DAPI
TUNEL
MERGE
NT
Dexa 0,1M
DNasi
CTRL

Figure 22. Representative images to show TUNEL-positive nuclei (red colour) relative to DAPI-positive nuclei (x40 magnification). On each line, TUNEL-assay staining of the three different conditions (NT: not treated; Dexa 0.1 uM and DNAsi as positive control). A TUNEL-positive nucleus is marked with a yellow arrow in AT-39. No TUNEL-positive cells were counted in both untreated A-T and CTRL sample. DNAsi treatment for 10 minutes was used as positive control of TUNEL assay.

On the other way, DAPI staining displayed a marked difference between AT-39 and CTRL in terms of DNA amount in the nuclei (Fig. 23 and table 5). In AT-39, the nuclei appeared larger and darker. Nuclear shape was circular vs. spindly. These differences reflect cell-cycle phase: most A-T cells are in G1, while the majority of CTRL cells are in S/G2/M with a higher amount of DNA (DAPI staining brighter) (Fig. 22; white arrow indicates a dividing cell). These differences increased after dexamethasone treatment (Fisher Test: p-value < 0.01), suggesting the drug is reducing cell proliferation in A-T cells.

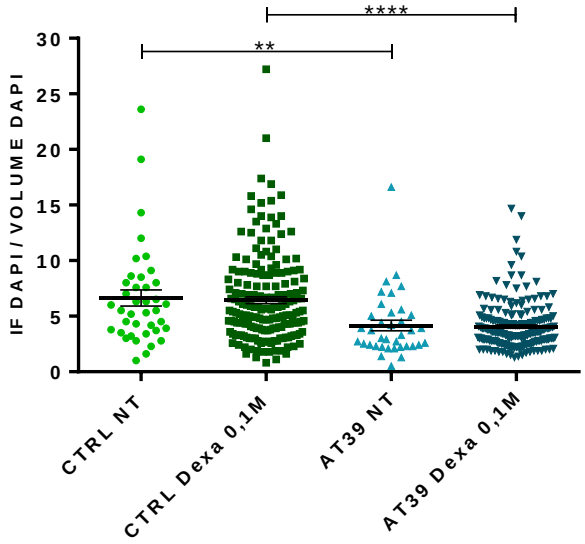


Figure 23. DAPI staining after dexamethasone treatment in A-T fibroblasts, displayed a marked difference between AT-39 and CTRL in terms of different DNA amount in the nuclei, normalized on cellular volume.

5. NF-kB activation after TNF-a and LPS stimulation.

Because glucocorticoids are mainly anti-inflammatory drugs, we decided to further study the canonical pathways associated with anti-inflammatory response activated by dexamethasone. We evaluated NF-κB as this is a major transcription factor that plays a key role in inflammation, apoptosis and oncogenesis (Kumar et al. 2011).

NF-kB activation was analyzed by western blot of nuclear extracts obtained from six A-T fibroblast cell lines and from three healthy controls, both at basal levels and after

treatment with LPS (1 µg/mL) or Tumor Necrosis Factor-α (TNF-α) (20 ng/mL) for 3 hours (Fig. 24). After LPS treatment, we showed a significant lower or absent phospho-p65 NF-κB formation in all A-T cells as compared to controls.

Using TNF-α, we obtained more variable results: in four cases we had a phospho-p65 comparable to controls, whereas in two we observed a decreased phospho-p65 (Fig. 24).

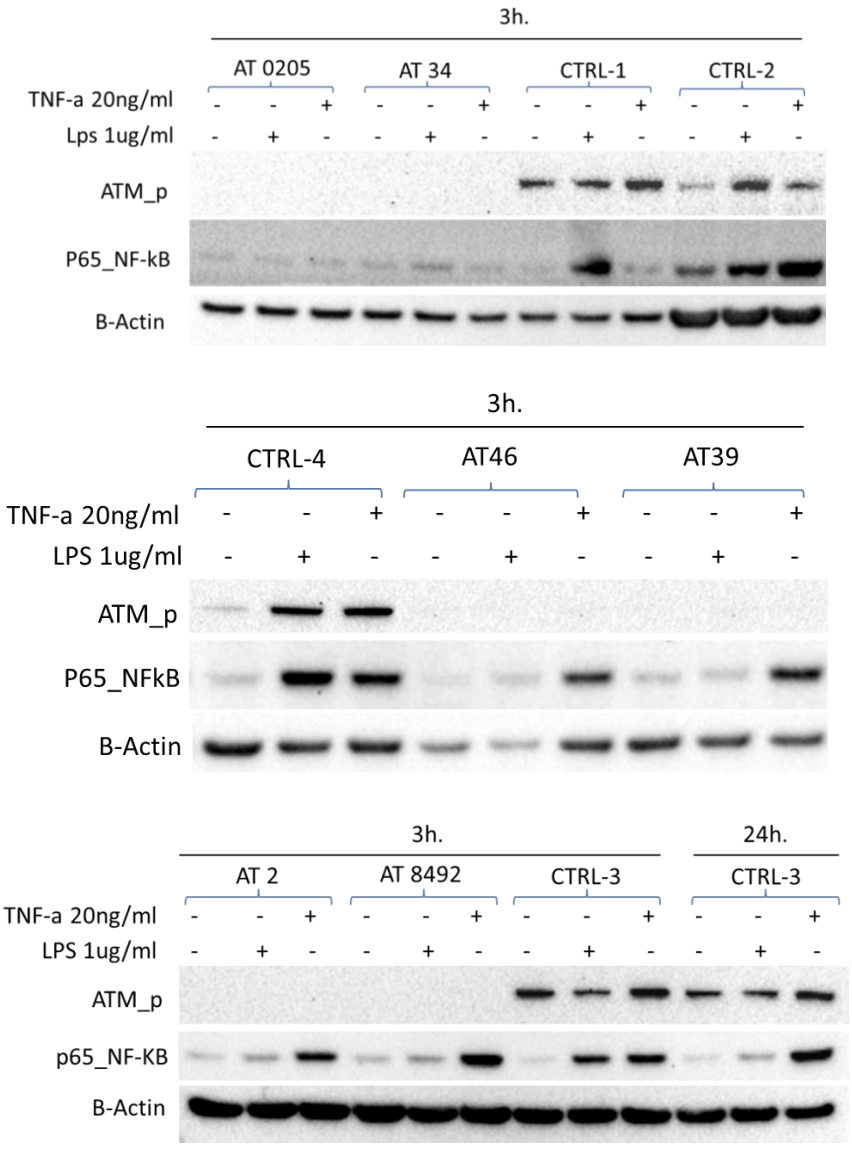


Figure 24. NF-kB activation in A-T cell lines.
Equal amounts of nuclear extract were analysed by WB to detect the level of p65 subunit of NF-Kβ. Following LPS treatment ATM is phosphorylated (p-ATM). Beta-actin was measured as loading control.

6. Evaluation of TLR-4, IL-6 and IL-8 gene expression.

Consequently, we hypothesized that the absence of NF-κB activation could be related to a defective PRRs-mediated response, namely TLRs. These receptors are known to mediate a rapid activation of NF-κB, which in turn induces pro-inflammatory mediators such as IL-6 and IL-8. According to the absence of NF-kB activation, we found a complete absence of TLR-4 and IL-6 gene expression in A-T cells, as compared to controls, both at basal levels and after LPS stimulation. In particular, TLR-4 expression did not change in A-T cells (over a time course of 30 hours), whereas IL-6 expression had only a modest increase after 3 hours, then declining to basal levels (Fig. 25).

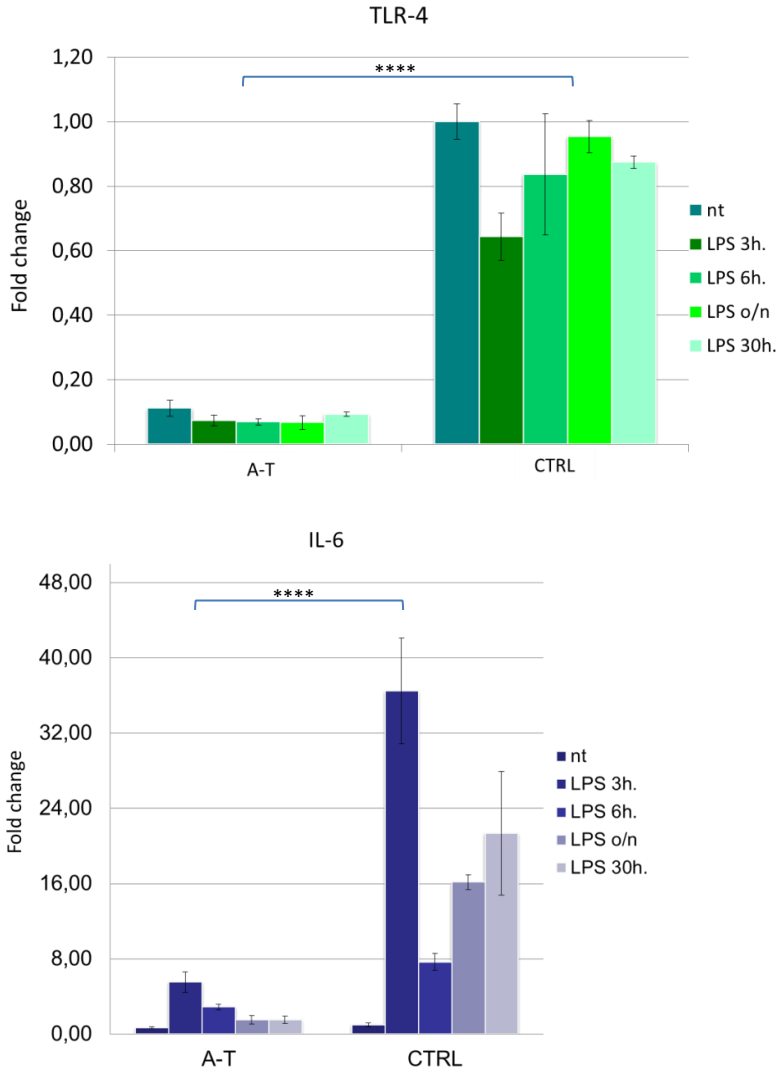


Figure 25. Quantitative RT-PCR data revealed differences in gene expression levels of TLR-4 and IL-6 in A-T cells. Compared to control cells, A-T cell lines showed a reduction of TLR-4 and IL-6 with and without LPS stimulation. TLR-4 is a receptor specific to LPS.

We measured IL-6 protein levels as secreted in tissue culture media after LPS treatment (48 hours). The concentration of IL-6 was significantly lower in the medium from A-T cells compared to controls at basal levels. Exposure to LPS resulted in a significant increase of IL-6 in A-T cells compared to controls, which exhibited a 10-fold raise (Fig. 26).

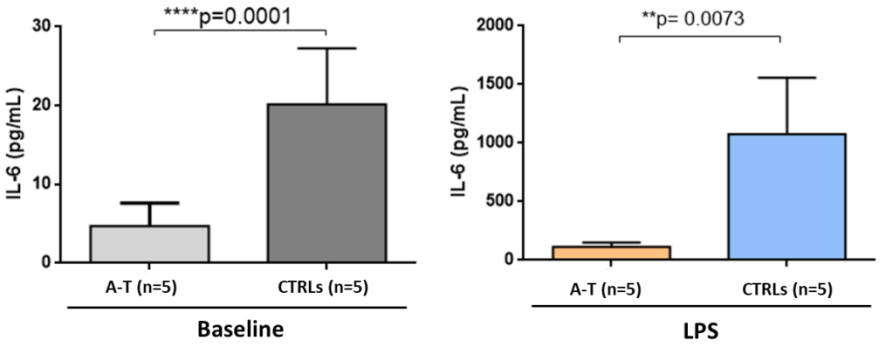


Figure 26. IL-6 measure in supernatant of A-T cell colture.
The range of IL-6 levels in A-T cells compared to CTRLs was significantly reduced, both in untreated and LPS-treated cells.

To confirm the absence of TLR-4 gene expression seen in A-T fibroblast cell lines, we showed its levels were drastically reduced also in an *ATM*-knockout HeLa cell, generated by CRISPR-Cas9 technology (Fig. 27).

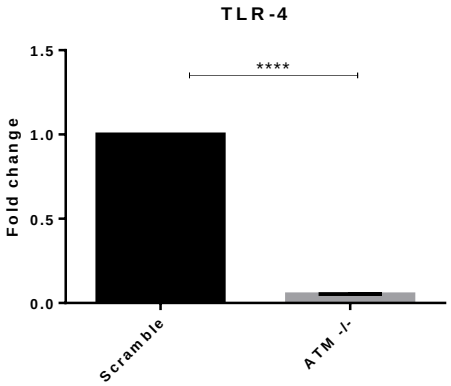


Figure 27. Quantitative RT-PCR data revealed in HeLa ATM$^{-/-}$ a marked decrease in gene expression levels of TLR-4. Compared to scramble HeLa control cells, HeLa ATM $^{-/-}$ cells showed a reduction of TLR-4 at basal level confirming results obtained in A-T fibroblast cell lines.

Next, we measured IL-8, a pro-inflammatory cytokine. Unstimulated A-T cells showed higher levels of IL-8 gene expression compared to CTRLs (Fig. 28). TNF-α or, more markedly, LPS stimulation were not able to raise IL-8 gene expression as in control. In particular IL-8 expression levels had only a modest increment after 4 hours of LPS stimulation compared to control lines, confirming a defective TLR-4 response pathway.

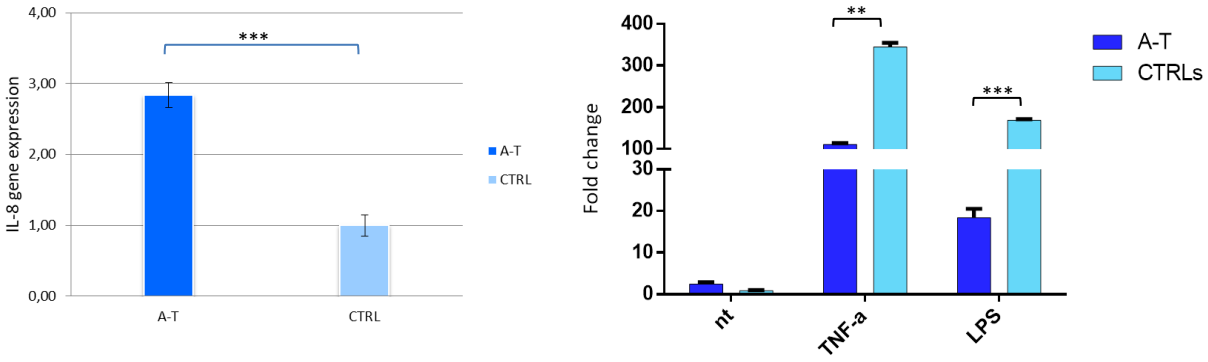


Figure 28. Quantitative RT-PCR analysis of IL-8 gene. We showed IL-8 gene expression was increased at basal levels in A-T cells. Four-hour stimulation with LPS or TNF-α is defective in inducing IL-8 expression in A-T cells. This impairment is particularly evident after LPS stimulation.

The increased levels of IL-8 in A-T cells revealed a chronic inflammatory state.

To investigate the anti-inflammatory effect of dexamethasone on IL-8 level, we performed qRT-PCR on A-T fibroblasts after dexamethasone treatment. We showed that dexamethasone dramatically reduced IL-8 expression. Unstimulated A-T cells have a 5-fold increase of IL-8 compared to control, whereas its level is comparable to control after dexamethasone treatment (Fig. 29).

To corroborate these data, cells were contemporary treated with dexamethasone and TNF-α, as an inflammatory stimulus. Dexamethasone decreased IL-8 gene expression at comparable levels both in A-T and CTRLs cells (Fig. 30).

These results showed dexamethasone was able to act as anti-inflammatory molecule reducing pro-inflammatory cytokines in A-T cells.

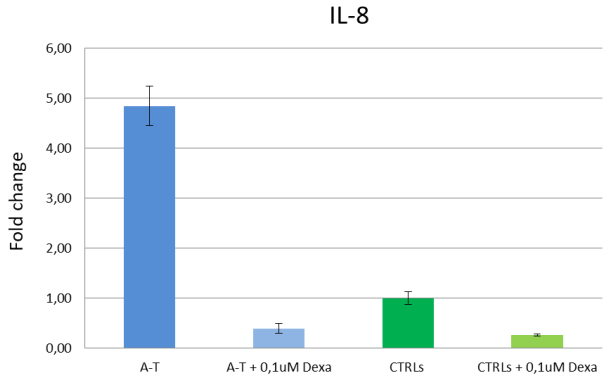


Figure 29. Expression analysis of IL-8 gene in A-T fibroblasts after dexamethasone treatment.
Quantitative RT-PCR was performed on A-T (n= 3) and CTRLs (n= 3) fibroblasts cell lines after 6-hour 0.1 µM dexamethasone treatment.

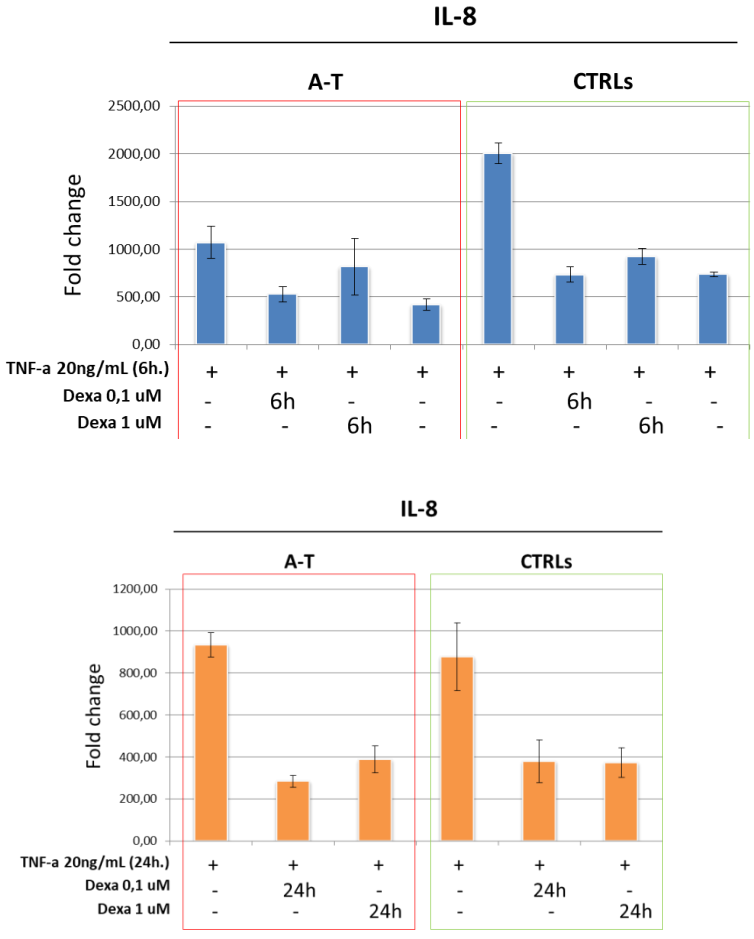


Figure 30. IL-8 expression analysis in A-T cell lines after dexamethasone and TNF-α induction.
Quantitative RT-PCR was used to measure IL-8 expression in A-T and control cell lines treated with dexamethasone (0.1 uM and 1 uM) and TNF-α at 6h (upper panel) and 24h (lower panel).

7. CD14 and TLR-2 protein analysis.

As LPS recognition by TLR-4 is strictly mediated by the expression of CD14, its co-receptor, we evaluated CD14 protein levels in our fibroblast cell lines before and after LPS treatment. Despite TLR-4 downregulation, CD14 levels did not show a significant decrease in A-T cells as compared to controls, both at basal levels and after LPS stimuli (Fig. 31, right panel).

We finally investigated TLR-2 expression, that is known to respond to bacterial lipopeptides, with a signaling cascade involving NF-κB. As for CD14, TLR-2 expression did not change significantly between A-T and control cell lines, although A-T cell lines had a slightly lower expression of TLR-2 (Fig. 31). TLR-2 gene expression levels were also measured by quantitative real-time PCR (qRT-PCR) (data not shown), confirming our flow cytometry analysis.

Overall, our data suggest that A-T derived cell lines exhibit a loss of expression of TLR-4 and a consequent lack of response when challenged by LPS in terms of pro-inflammatory cytokines (IL-6 and IL-8), likely resulting in an impaired innate immunity response to bacterial infections.

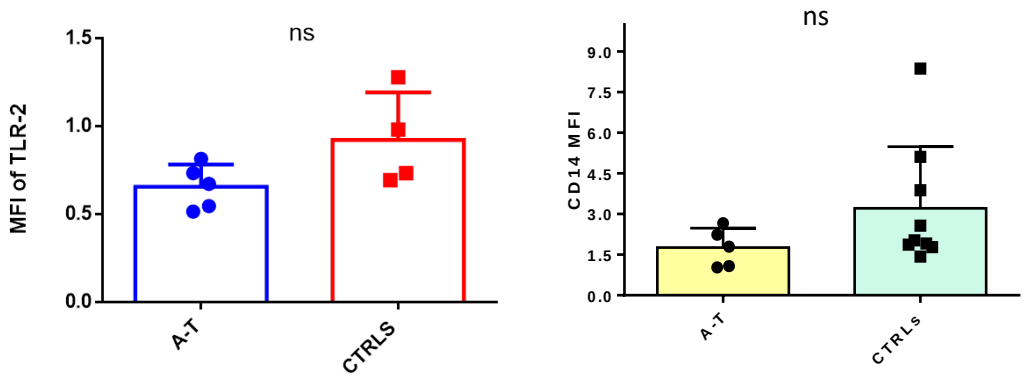


Figure 31. TLR-2 and CD14 protein levels in A-T cells.
In A-T fibroblasts, TLR-2 and CD14 levels are not significantly different from controls, although both have a tendency to reduction.

Chapter 5
Discussion

Currently there is no line of therapy for treatment or prevention of A-T. Certain approaches that are being used are only able to slow down the progression of the disease and alleviate some of its symptoms (Guleria et al., 2016). Recent evidence reported that synthetic glucocorticoids, such as dexamethasone, might yield short-term mild improvement in A-T neurological symptoms and motor coordination (Broccoletti et al., 2008; Russo et al., 2009; Broccoletti et al., 2011; Zannolli et al., 2012; Chessa et al., 2014; Leuzzi et al., 2015).

Our aim was to study the effect of glucocorticoids in A-T cells, starting from recent *in vitro* studies showing that dexamethasone promotes the formation of a non-canonical mRNA (ATMdexa1) encoding for a functional "mini-ATM" protein (Menotta et al 2012).

Critical reading of these data revealed an unconceivable lack of scientific robustness.

First, *ATM* variants in databases (e.g., ENSEMBL) do not report such a transcript. Second, the generation of ATMdexa1 implies a splicing between exon 3 and exon 52, not mediated by the canonical splicing machinery. Indeed, splicing between exon 3 and 52 is suggested to be mediated by a repeat, in a mechanism referred by the authors as SDR-splicing, reported as a rare event in plants and never described in Metazoa (Fan et al., 2007). Third, the generation of "mini-ATM" protein requires a non-canonical ATG that starts the translation from an internal site located in exon 57 (Met 2806). This ATG would produce an ATM variant protein starting from codon 8,418 of the canonical ATM cDNA. (Menotta et al 2012). No explanation is given about the molecular mechanism by which the new mRNA variant drives the start of translation: the transcript itself should contain an internal ribosome entry site, permitting the translation of the ATMdexa1 RNA into the miniATM protein. It is not clear if the transcript is not expressed in wild-type cells or the mini-ATM protein is not translated (Menotta et al., 2012). Fourth, the presence of a functional mini-ATM is in contrast with the notion that the N-terminus of the protein, which carries nuclear localization sequences and binding sites for chromatin and ATM-interacting protein (Young et al., 2005). Because the newly discovered ATM variant induced by dexamethasone lacks the N-terminus, it is expected to be unable to exert the full nuclear function. Young et al. (2005) reported that mutagenized ATM proteins lacking the N-terminus are unable to fully localize in the nucleus and activate the DNA repair effectors. Nevertheless, dexamethasone has been shown to increase the production of

GSH and NADPH, which improved the antioxidant capacity of A-T cells to counteract oxidative stress, having the mini-ATM protein (Biagiotti et al., 2016).

To investigate better this “unusual” mechanism, we performed the same experiments in our survey of patients, initially using LCLs. All except two (AT2 and AT38), carried mutations that fall inside the ATMdexa1 expected transcript, as the three cases described in Menotta et al. 2012. In none of our six cell lines we found ATMdexa1, which was also absent in primary dexamethasone treated A-T fibroblasts. Furthermore, a qPCR assay, specific for ATMdexa1, failed to reveal any minimal amount of this alleged transcript, both in LCLs and fibroblasts.

Given these data, we cannot but conclude ATMdexa1 and the mini-ATM protein are technical artifacts.

However, we decided to further investigate the functional data showed by the authors to provide alternative explanations to their experiments, and possibly further elucidate the role of glucocorticoids in A-T cells.

We studied both nuclear and cytoplasmic ATM roles. In ATM-dependent DNA damage response pathway, we showed that, both at basal level and after ionizing radiation, H2AX activation was enhanced (~35%) by dexamethasone both in controls and A-T LCLs. This result was partially explained by the fact that H2AX is known to be phosphorylated both by ATM and DNA-PK, which act redundantly in DSBs response (Serrano et al., 2012; Chen et al., 2011). Indeed, the specific inhibition of DNA-PK kinase activity, showed a marked decrease of γH2AX, suggesting its phosphorylation depends upon DNA-PK, and it is enhanced by dexamethasone.

The high level of basal γH2AX was initially unexpected in LCLs, and may be related to the high rate of division that accumulate DSBs, or to the EBV transformation that introduce gene expression changes. In a recent report by Zijno et al, (2010) DNA repair capacity was compared between PBMCs and EBV-transformed LCLs derived from twenty healthy subjects by analyzing γH2AX foci formation assay and micronuclei test. The authors observed that larger inter-experimental variability occur in the results obtained with LCLs compared to PBMCs (Zijno et al., 2010). In addition, they have shown LCLs are an inappropriate model system for DNA repair studies with cell-free based functional assays (Hussain et al., 2012; Mazzei et al., 2011).

For these reasons, we changed our model system to primary A-T skin fibroblasts. We showed that dexamethasone treatment was able to increase γH2AX only after IR but not at basal levels despite the data collected in LCLs. Moreover, after NU7441 treatment γH2AX levels decreased at very low levels, confirming that in cells lacking the ATM protein, the activation of H2AX is DNA-PK-dependent.

To follow further the DNA-PK pathway, we studied PKB/AKT. There is broad evidence that GC possess a cell-type specific, dual role in the regulation of programmed cell death (Amsterdam et al., 2002). In a variety of hematopoietic cells such as monocytes, T-lymphocytes and lymphoma cells GC are able to induce apoptosis (Distelhorst et al., 2002; Planey et al., 2000;Schmidt et al., 2004). Conversely, recent studies indicate that GC can inhibit apoptosis in fibroblasts mediated by the PKB/AKT signaling cascade (Sapolsky et al., 2000). At basal level, PKB/AKT is stimulated by growth factors, hormones and other extracellular stimuli. *In vitro*, a strong basal activation PKB/AKT is detectable in control fibroblasts but not in A-T cells. This is due to the cytoplasmic role of ATM, which directly phosphorylates ser-473 of PKB/AKT in response to extracellular stimuli (Yang et al., 2011).

Halaby at al. (2008) showed that ATM inhibition by KU55963 in L6 myoblasts resulted in abrogation of AKT phosphorylation at Ser-473 as well as a dramatic reduction of insulin mediated glucose uptake.

We showed the dexamethasone effect on fibroblasts is strongly dependent upon ATM, although a residual activation of p-AKT is present in A-T cells. This effect is particularly evident in fibroblasts challenged with both dexamethasone and IR, where the p-AKT increase is clearly lower than in controls, and it is likely due to DNA-PK as this was previously shown to mediate H2AX phosphorylation in the absence of ATM.

ATM has an important role in regulation cellular defenses against redox activity (Reliene et al., 2007 and 2008; Bencokova et al., 2009). Indeed, this role of ATM seems critical for the neurodegeneration, and has suggested the use of antioxidant therapy for management of A-T (Reliene et al, 2008; Schubert et al., 2004).

We have also provided support that in A-T cells there is an altered ROS homeostasis. In a recent paper, we demonstrated that in peripheral blood of A-T patients there were alterations in the essential trace elements copper and zinc, which are involved in the

oxidative stress response (Squadrone et al., 2015). This was associated with transcriptional and functional alterations of ROS-detoxifying enzymes (CAT, SOD1, SOD2 and GPX1) in A-T LCLs.

Based on this evidence, we investigated if dexamethasone could have an effect in restoring the redox imbalance present in A-T cells. Dexamethasone treatment was able to reduce Glutathione Peroxidase (GPX1) gene expression, even if never reach levels comparable to controls. However, we noticed a great variability between the different A-T cell lines possibly due to different *ATM* mutations or more likely to a compromised oxidative stress state in A-T cells, associated with excessive levels of ROS species. Taken together, our results demonstrated that, if on one hand dexamethasone could have an effect in some ATM dependent pathways bypassing the lack of ATM and increasing some direct and indirect ATM substrates, on the other hand it could not efficiently act in a detoxifying pathway. This was confirmed when we evaluated intracellular ROS levels after 72 hours of dexamethasone stimulation. We showed that A-T cell lines had constitutive higher levels of intracellular ROS compared to healthy controls, and these levels increased after treatment, suggesting an adverse effect, an opposite finding to that reported in the literature even in A-T lymphoblasts (Biagiotti et al., 2016).

ROS increase after dexamethasone treatment was indeed apparent both in A-T and control cell lines, even at low doses. Moreover, we found that in A-T fibroblasts, dexamethasone stimulation for 3 days induced a significant cell cycle arrest in G1 phase, a possible deleterious effect that could defeat the anti-oxidant defense system and induce genome instability.

The pro- or anti-apoptotic outcome of glucocorticoid signaling is highly dependent upon the cell type receiving the signal (Yates et al., 2013). Glucocorticoids are a widely prescribed class of drugs used to combat inflammation and disease, and are often used in the treatment of different types of tumors.

Mechanistically, glucocorticoids seem to influence many signaling pathways such as those initiated by death receptors, mitochondria, proteasomes, lysosomes, PI3-K/AKT, NF-kB, SGK-1, MKP-1, JNK, Wnt, GSK3 and tissue formation, which together may contribute to glucocorticoid-mediated pro-or anti-apoptotic effects, dependent on the cellular state.

Published data overall suggest to investigate the canonical role of dexamethasone as anti-inflammatory drug. Indeed, recent studies have provided evidence for an inflammatory

phenotype in A-T (McGrath-Morrow SA et al., 2010) which has been linked to a defect in the innate immune response (Hartlova et al., 2015; Erttmann et al., 2016). Recurrent upper and lower respiratory tract infections may progress to bronchiectasis and interstitial lung disease characterized by fibrosis and chronic inflammation. Respiratory disease causes significant morbidity and mortality in patients with A-T, with up to 60% of deaths due to lung complications (Erttmann et al., 2016; McGrath-Morrow et al., 2010).

Glucocorticoids are widely used due to their potent anti-inflammatory and immunosuppressive effects. However, the molecular mechanisms behind these effects are not fully understood, although several targets have been identified. Since glucocorticoids are known to interfere with many signaling pathways and molecules involved in TLR signaling (Mogensen et al., 2008; Hermoso et al., 2004), it has been hypothesized that TLR signaling pathways may be important targets for glucocorticoid action and may explain many of their anti-inflammatory and immunosuppressive effects.
We demonstrated a significant lower or absent phospho-p65 NF-κB formation after LPS treatment in all A-T patients as compared to controls, whereas only minimal changes were observed following TNF-α treatment, in four out of six A-T patients. NF-κB activation, a major transcription factor that plays a key role in inflammation, apoptosis and oncogenesis (Kumar et al. 2011).
Consequently, we hypothesized that the absence of NF-κB activation could be related to a defective PRRs-mediated response, namely TLRs. The signal transduced from these receptors are known to lead to a rapid activation of NF-κB, which prompts the production of pro-inflammatory mediators such as IL-6 and IL-8. Both at basal levels and after LPS stimulation, we found the absence of NF-kB activation and IL-6 secretion. Accordingly, TLR-4 and IL-6 gene expression were strongly downregulated. IL-8 was significantly up-regulated compared to control cells. However, after 4 hours of LPS and TNF-a treatment, we found a reduced IL-8 gene expression in A-T cells as compared to control cell lines, both after TNF-a stimulation and LPS stimulation.
Regarding TLR-4 expression levels did not change in A-T cells (over a time course of 30 hours), whereas IL-6 expression had only a modest increase after 3 hours, then declining to basal levels afterwards. This correlates with a minimal increase of IL-6 secretion

measured in supernatants of A-T cells as compared to control cells that exhibited a significant higher increase after 48 hours of LPS stimulation.

These observations highlighted the impaired LPS induction in A-T cells, suggesting that a defective of TLR-4 (known to be specific to binding LPS), but not its co-receptor CD14, could impair the innate immune response to immunogenic challenges.

Patter Recognition Receptor (PRRs) as TLR-2 and TLR-4 are very poorly studied in the pathogenesis of A-T. However, their role have recently been established in the pathogenesis of several chronic inflammatory diseases in animal models, and specifically TLR-4 polymorphisms are associated with several human age-related diseases, including atherosclerosis, type 2 diabetes, and rheumatoid arthritis, raising the question of whether these receptors also contribute to inflammatory programs associated with neurodegenerative disease (Balistreri et al., 2009).

Repeated activation of TLR signaling results in a reduction in the subsequent pro-inflammatory cytokine response, a phenomenon known as TLR tolerance (Peroval et al., 2013), as well as changes in expression of other TLRs, known as cross-tolerance (Calvano et al., 2003).

Cytoplasmic membrane-associated TLRs signal through two primary pathways, defined by the adaptor molecules used to initiate each signal cascade. The classical MyD88-dependent pathway relies on functioning cell surface TLRs, is common to both TLR-2 and −4, and leads to rapid activation of NF-κB to induce pro-inflammatory mediators such as TNF-α, IL-6 and COX-2 (O'Neill et al., 2013).

Our results support severely deficient MyD88-dependent TLR signaling in A-T cells, based on the observations of (1) decreased gene expression of TLR-4 (2) diminished response under LPS challenge of MyD88-dependent pro-inflammatory mediators, such as IL-6 and IL-8.

Interleukin-6 is a cytokine not only involved in inflammation and infection responses but also in the regulation of metabolic, regenerative, and neural processes. IL-6 is considered a neuropeptide capable of inducing neuronal differentiation and the results obtained on our A-T fibroblast cell lines could explain in part the pathogenesis of A-T.

IL-8 can be secreted by any cells with toll-like receptors that are involved in the innate immune response. Interleukin-8 is a key mediator associated with inflammation where it plays a key role in neutrophil recruitment and neutrophil degranulation (Harada et al.,

1994). As an example, it has been cited as a proinflammatory mediator in gingivitis (Haake et al.2002), and psoriasis. Interleukin-8 secretion is increased by oxidant stress, which thereby cause the recruitment of inflammatory cells and induces a further increase in oxidant stress mediators, making it a key parameter in localized inflammation.

The neurological abnormalities and/or neurodegeneration of Purkinje cells in A-T patients is not yet known (Hoche et al., 2012). Data support the idea that a defective response to genotoxic and/or oxidative stress contributes to the neuronal cell dysfunction and death in A-T. This may be due to impaired trafficking or proper localization of this receptor on the surface membrane or to other related unknown mechanisms, likely resulting in an impaired innate immune response to bacterial infections.

The validation of the anti-inflammatory effect of dexamethasone on IL-8 gene expression levels at different concentrations (0.1, 1,10 uM) after TNF-a stimulation at 6 hrs and 24hrs in both cell lines prompt us to found differences between A-T fibroblasts and controls cells in term of activation of IL-8 gene expression: at 6h hours after TNF-a stimulation, IL-8 levels of A-T cells were significantly lower than controls, but at 24 hours IL-8 levels in A-T patients are comparable to those of controls, suggesting that response to the TNF-a treatment was present in most A-T patients as we have seen in NF-KB's western blot results, conversely to LPS stimulation that was significantly lower in A-T patients compared to controls.

In conclusion, our results suggest that:

- Literature data on ATMdexa1 and miniATM suffer from technical artifacts and wrong conclusion derived from the selection of wrong cellular models such as LCLs.
- The role of dexamethasone as treatment in A-T neurological symptoms is hardly explained by the increase in DNA-PK activity which partially, but very mildly vicariates ATM. This reasoning must also be put in place with the very low dosage of dexamethasone administer to patients that may reach brain.
- Dexamethasone may have a role increasing oxidative stress, which in specific sensitive tissues may at long term, worsen the pathology. However, this issue needs further studies, likely in animal models, to be solved.

- The state of chronic inflammation present in A-T patients and at the basis of recurrent sinopulmonary infections by bacteria and viruses could be explained in a defective activation of NF-kB, one of the key molecules that act in the innate immune response activation pathway, and of the PRRs and TLRs receptors involved in the cell membrane recognition of different bacterial peptides.

Dexamethasone treatment should be carefully considered both due to its known immunosuppressant effects and to its role enhancing ROS in vitro. Moreover, the discovery in A-T cell fibroblasts of deficient activation of some molecules that play an important role in the activation of the innate immune pathway response to infections, could set the basis for investigating a new role of *ATM* in innate immunity.

Further studies along this line could open a new scenario for the discovery of novel therapeutic intervention in A-T disease.

Chapter 6
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