*Communication*

# SMN Deficiency Destabilizes ABCA1 Expression in Human Fibroblasts. Novel Insights in Pathophysiology of Spinal Muscular Atrophy.

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**Abstract:** The deficiency of Survival Motor Neuron (SMN) protein causes Spinal Muscular Atrophy (SMA), a rare neuromuscular disease that affects different organs. SMN is a key player in RNA metabolism regulation. An intriguing aspect of SMN function is its relationship with plasma membrane-associated proteins. Here, we provide a first demonstration that SMN affects the ATP-binding cassette transporter A1, (ABCA1), a membrane protein critically involved in cholesterol homeostasis. In human fibroblasts, we showed that SMN associates to ABCA1 mRNA, and impacts its subcellular distribution. Consistent with the central role of ABCA1 in the efflux of free cholesterol from cells, we observed a cholesterol accumulation in SMN-depleted human fibroblasts. These results were also confirmed in a SMA type I patient-derived fibroblasts. These findings not only validate the intimate connection between SMN and plasma membrane-associated proteins, but also highlight a contribution of dysregulated cholesterol efflux in SMA pathophysiology.

**Keywords:** SMN; SMA; plasma membrane; ABCA1; cholesterol

## 1. Introduction

The Survival Motor Neuron (SMN) protein influences the RNA life cycle and RNA-related pathways in all eukaryotic cells [1-3]. SMN protein is encoded by two almost identical genes, named SMN1 and SMN2, located on chromosome 5q13 of the human genome. These genes differ by only five base pairs, remarkable, by a single nucleotide within the coding sequence (C > T). The centromeric SMN2 gene primarily produces alternatively spliced transcripts encoding defective SMN protein. Consequentially, SMN2 cannot fully compensate for SMN1 alterations (unless SMN2 is present in multiple copies). Deletions or, less frequently, mutations of the telomeric SMN1 gene represent the diagnostic parameter of spinal muscular atrophy (SMA), one of the most common pediatric genetic diseases [4, 5]. SMA is classified into four different subtypes (SMA type I-IV), based on age of onset and clinical severities. The spectrum of SMA also includes the type 0 variant that identifies cases in which the degenerative process can already be detected during late stages of pregnancy [6]. Degeneration of alpha-motoneurons in the spinal cord and a progressive muscle weakness, are the main clinical manifestations observed in the most severe form of disease. Although the large alpha-motoneurons in the spinal cord display the highest susceptibility, SMN deficiency perturbs additional tissues and organs [7, 8]. This is consistent

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with the fact that SMN drives multiple facets of RNA metabolism, ranging from transcription to local translation, in all cell types [2, 3]. Our idea is that a large cell surface extension and polarity can exacerbate the adverse consequences of SMN deficiency. In this context, an intriguing aspect of SMN function may be its physical and functional link with plasma membrane (PM) compartments. In human fibroblasts, we showed that SMN binds to structural components of the PM, such as caveolin-1, and relocalizes at lipid rafts microdomains during active cytoskeleton dynamics [9]. Interestingly, SMN is required to establish proper cell polarity by the accurate synchronization, in time and space, of actin filaments polymerization and protein synthesis. Moreover, our previous studies reported that SMN affects membrane composition as well as the association of distinct transcripts to the PM [10], which emerges as a docking site for RNA-protein complexes [10, 11]. These findings prompted to understand if exists an implication of SMN in composition and metabolism of the PM. Indeed, a study by Deguise and colleagues, showed an increased propensity to developing dyslipidaemia and liver steatosis in patients with SMA [12]. Notably, this work is in line with early studies identifying defects in fatty acid oxidation and lipid metabolism in SMA [13-15]. In the era of SMA therapy a deep understanding of the molecular mechanisms underlying this severe pathology is crucial to provide more clinical benefits, we further focalized on the intriguing relationship between SMN and PM networks.

Cholesterol is a major lipid constituent of the plasma membrane contributing to membrane integrity and fluidity; it plays an essential role in several biological process [16-18]. Due to its unique ability to interact with phospholipids, cholesterol influences membrane microdomains, membrane trafficking, and signal transduction. Importantly, cholesterol can also interact with membrane proteins affecting their function. Dysregulated cholesterol homeostasis is implicated in several human pathologies, including cancer, cardiovascular and neurodegenerative disease [19, 20]. Mammalian cells have developed sophisticated mechanisms to prevent abnormal accumulation of cholesterol in cellular membranes [21].

Energy-dependent efflux of cholesterol from cells is mediated by ATP-binding cassette (ABC) transporters, primarily ABCA1. ABCA1 is a membrane-associated protein that acts to transfer intracellular cholesterol to apoliproteins (ApoA-I) promoting the formation of nascent high-density lipoproteins/HDLs [21-23]. Mutations in ABCA1 have been associated with Tangier disease, a rare genetic disorder characterized by extremely low levels of HDL and apoA-I; excessive deposition of cholesteryl esters in macrophages, and lipid deposits in cells including fibroblasts, Schwann cells, and myofibers [24-27]. ABCA1 is expressed ubiquitously. However, in some tissues there is a discrepancy between ABCA1 mRNA and protein expression, suggesting the existence of regulatory mechanisms at both transcriptional and post-transcriptional levels [28].

Here, we provide a first demonstration of a relationship between SMN and the membrane-associated protein ABCA1. In human fibroblasts, we showed that SMN associates to ABCA1 mRNA, and affects its subcellular localization. Consistent with the pivotal role of ABCA1 in cholesterol efflux from cells, we visualized an accumulation of cholesterol in SMN-depleted cells, compared with the control. Importantly, this result was confirmed in a SMA type I patient-derived fibroblasts. These findings further confirm an intimate connection between SMN function and plasma membrane dynamics and point out that dysregulated cholesterol homeostasis might be a critical component in SMA pathophysiology.

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## 2. Results

### 2.1. *SMN associates to ABCA1 mRNA.*

Keeping in mind that SMN associates and impacts structural components of the plasma membrane [9-10], we deeply explored this intriguing feature of SMN starting to focalize on ABCA1, a major regulator of cholesterol homeostasis. First, we analyzed the localization pattern of endogenous ABCA1 mRNA in single cells. We subjected human fibroblasts to padlock assay, a method useful to visualize transcripts of interest with high selectivity [10]. By fluorescence microscopy, ABCA1 mRNA appeared located throughout the cytoplasm and nuclear/perinuclear regions of the cell (figure 1A). Interestingly, several fluorescent dots/amplicons were present at the cell periphery, indicating a traffic to and/or from the plasma membrane. Next, we combined ABCA1 padlock probe and SMN protein immunostaining. As shown in figure 1B, distinct subcellular sites showed the presence of overlapped fluorescent signals (yellow dots), suggesting a partial colocalization between ABCA1 mRNA and SMN protein. Most of the overlapped dots were detectable in nuclear/perinuclear sites. To note, some of these overlapped dots were present also in cell periphery, in close proximity to the plasma membrane. To provide a demonstration of a physical association between SMN and ABCA1 mRNA, we carried out RNA-immunoprecipitation (RIP) experiments. In agreement with fluorescence microscopy data, we revealed the existence of SMN protein-ABCA1 mRNA complexes in human fibroblasts (figure 1C). This novel interaction of SMN prompted us to suppose a SMN implication in ABCA1 modulation.

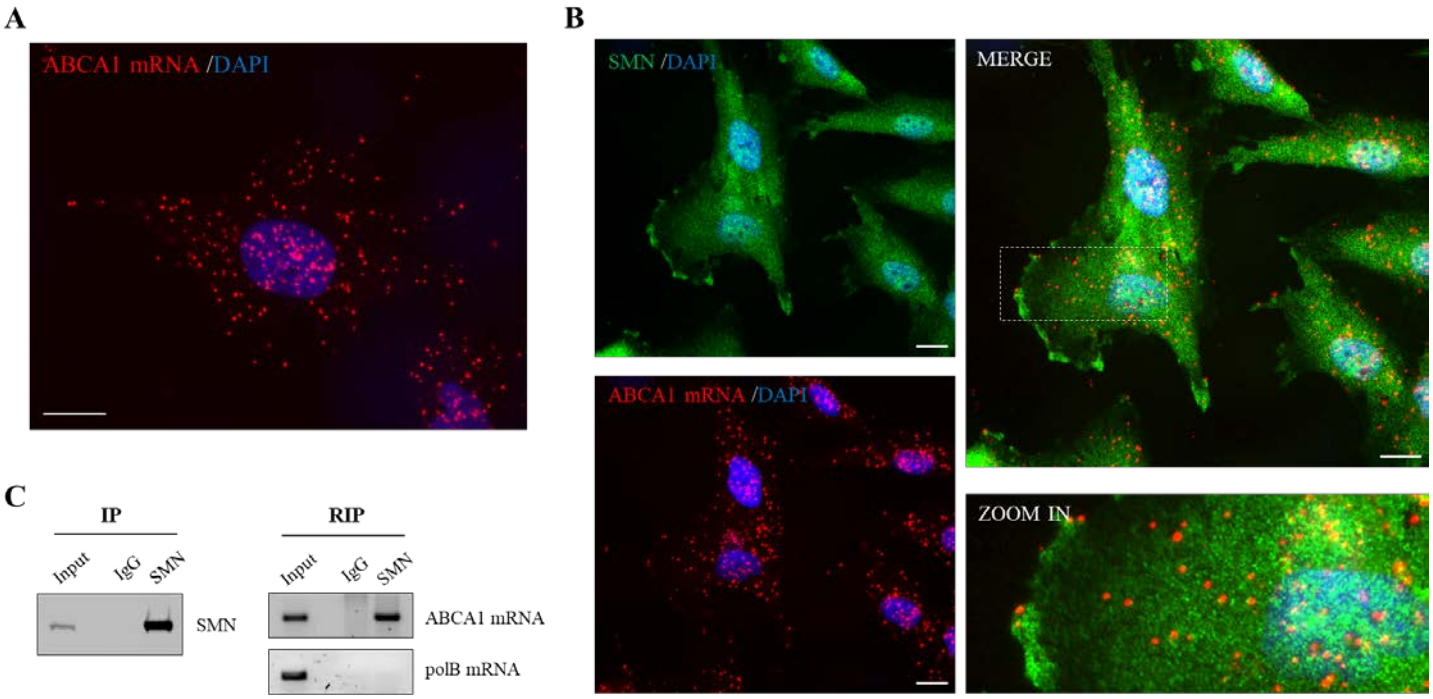


**Figure 1. SMN associates to ABCA1 mRNA.** (A) Representative image of human fibroblasts subjected to a padlock assay targeting ABCA1 mRNA. AlexaFluor 595-labelled probe allowed the detection of amplicons (red dots). Nuclei were labelled with DAPI (blue). Scale bars, 10 μm. (B) Fibroblasts were subjected to a combination of SMN protein immunostaining (green) and padlock assay targeting ABCA1 mRNA (red), and then were imaged by epifluorescence microscopy. Nuclei were stained with DAPI (blue). Scale bar, 10 ◎ m. Higher magnification of the boxed area (zoom in)

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reveals some of the overlapped fluorescent signals (yellow dots). (C) Cellular extracts were processed for RNA-immunoprecipitation (RIP) assay using SMN monoclonal antibody-conjugated beads. As negative control was used mouse IgG-conjugated beads. Immunoblotting validating the efficiency of SMN immunoprecipitation (left panel). ABCA1 mRNA presence in RIP samples was checked by semiquantitative (right panel). RT-PCR analysis of PolB mRNA was used as negative control. Panels are representative of three independent experiments.

### 2.2. *SMN knockdown affects the subcellular distribution of ABCA1 mRNA.*

The next step was to verify an implication of SMN in subcellular localization of ABCA1 mRNA. To this end, we down-regulated SMN expression levels by transfection of human fibroblasts with a pool of SMN1-selective siRNAs (siSMN). Scrambled siRNAs were used as control (siControl). 48 hours after transfection, fibroblasts were subjected to a padlock assay targeting the ABCA1 mRNA. A combination of padlock assay and SMN immunostaining was carried out to identify SMN depleted cells (figure 2A). In siControl-transfected cells, ABCA1 mRNA was found diffusely distributed throughout the cytoplasm, in peripheral as well as nuclear/perinuclear sites (figure 2A, siControl). Conversely, ABCA1 mRNA amplicons exhibited a propensity to accumulate at nuclear/perinuclear subregions upon SMN knockdown (figure 2A siSMN). By quantitative analysis of amplicons, we evaluated a general significant increase of ABCA1 transcript in SMN-deficient cells (figure 2B). Noteworthy, by padlock experiments we observed that SMN deficiency disturbed peripheral location of ABCA1 mRNA. Given our previous study, suggesting that SMN could promote membrane compartmentalization of a subset of transcripts [10], we wanted to verify: 1) whether a pool of ABCA1 mRNA could associate to plasma membrane compartments; 2) whether this association could occur in a SMN-dependent manner. To this end, we approached a biochemical method useful to isolate plasma membrane-enriched fractions (PMEFs) from cultured cells [9]. Both siSMN- and siControl-transfected fibroblasts were processed to obtain PMEFs. Then, total RNA was extracted from whole cell extracts (WCE) as well as their respective PMEFs. By a semiquantitative RT-PCR, we observed that ABCA1 transcript was detectable in WCE and, most importantly, in PMEFs of human fibroblasts (figure 2C). Remarkable, we found that SMN knockdown reduced the abundance of ABCA1 mRNA in the PMEFs, despite its higher detection in whole cells (figure 2D). Considering the assumption that the PM could act as a docking site for a subset of transcripts, we suggest that: 1) ABCA1 mRNA may be one of the membrane-associated transcripts; 2) ABCA1 mRNA membrane compartmentalization may be influenced by SMN.

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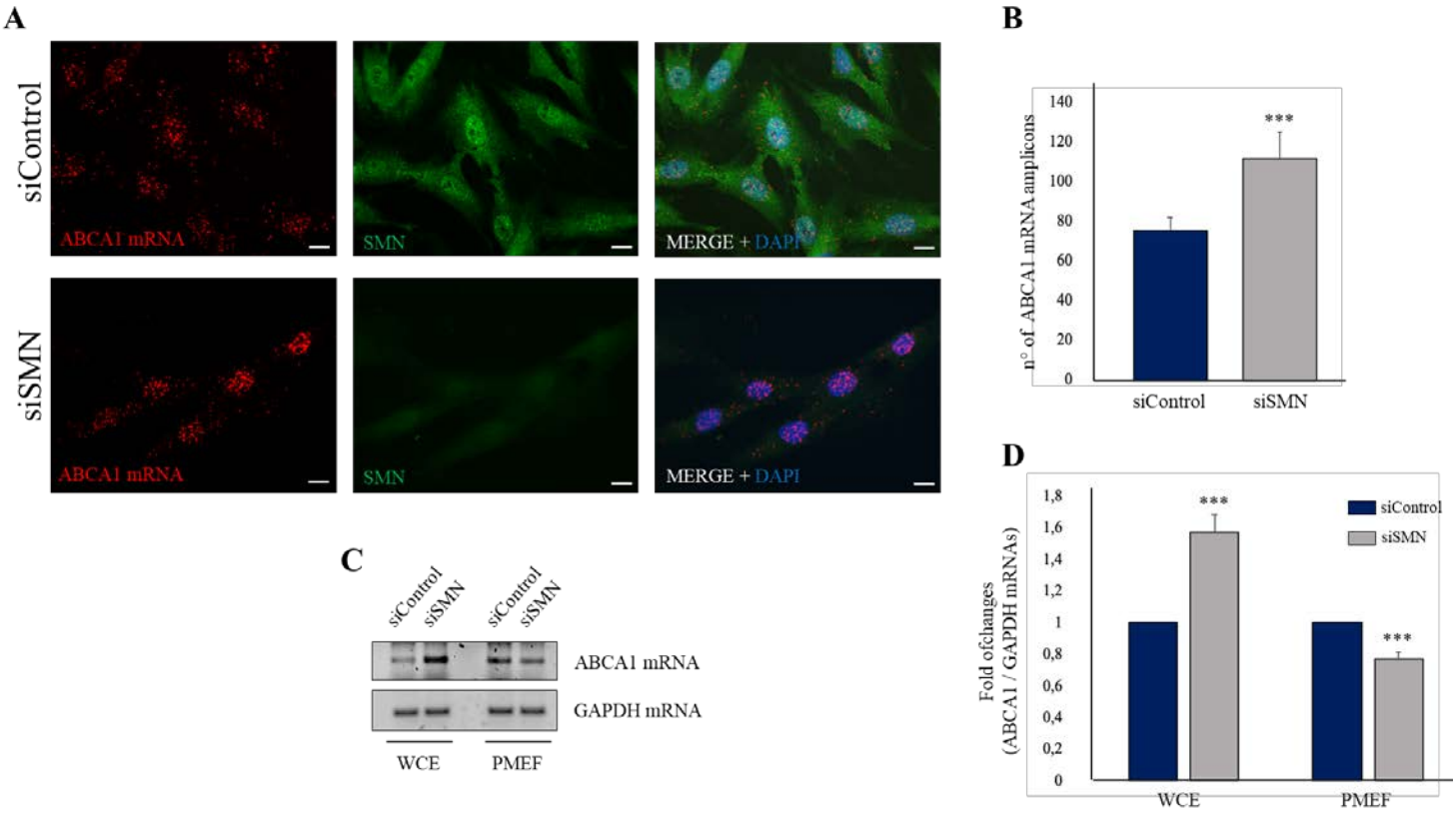


**Figure 2. SMN knockdown affects the subcellular distribution of ABCA1 mRNA.** (A) The combination of padlock assay targeting ABCA1 mRNA (red dots) and SMN protein immunostaining (green) was carried out in siControl- and siSMN-transfected human fibroblasts. Nuclei were stained with DAPI (blue). Scale bar, 10 ◎ m. (B) Quantitative analysis of ABCA1 mRNA amplicons per cell (n = 20 cells were analyzed for each condition). Data represent the mean of three independent experiments. Error bars represent s.d.. Asterisks indicate significative differences using unpaired t-test (*** $P < 0.01$). (C) ABCA1 mRNA and GAPDH mRNA were checked in WCE and PMEF of siControl- and siSMN-transfected fibroblasts by semiquantitative RT-PCR and analyzed by agarose gel electrophoresis. Panels are representative of three independent experiments. (D) Densitometric analysis of ABCA1 mRNA normalized to GAPDH mRNA in WCE and PMEF of siSMN transfected fibroblasts compared with the control (siControl). The graph illustrates the mean of three independent experiments. Error bars represent s.d.. Asterisks indicate significative differences using unpaired t-test (*** $P < 0.01$).

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2.3. *SMN deficiency downregulates ABCA1 protein levels and causes intracellular accumulation of cholesterol.*

Given the novel link between SMN and ABCA1 mRNA, we asked whether SMN could also influence expression levels of ABCA1 protein. By western blot analysis, we checked and compared ABCA1 protein content in siSMN- and siControl-transfected fibroblasts (figure 3A). Furthermore, since ABCA1 protein induction occurs in an ATP-dependent manner [26], we also monitored ABCA1 protein levels in cells stimulated by a standard ATP-depletion/recovery assay [9, 29, 30]. A band of approximately 254 kDa, corresponding to the molecular weight of ABCA1, was detected in unstimulated fibroblasts. In our system, ABCA1 protein appeared unchanged or slightly reduced in SMN-depleted cells, compared to control (figure 3A, unstimulated; Figure 3B). Interestingly, ATP-depletion/recovery stimulation caused an increase of ABCA1 protein, which was impaired in SMN-depleted cells (figure 3A, stimulated; Figure 3B). Thus, our findings demonstrate that SMN impacts ABCA1 mRNA intracellular distribution, and this correlates with an altered ABCA1 protein level. Given the pivotal role of ABCA1 in counteract cellular cholesterol accumulation, we suspected a disturbed distribution of cholesterol following SMN knockdown. To verify this hypothesis, we visualized intracellular cholesterol by subjecting fibroblasts to filipin staining. As shown in figure 3C (unstimulated), images from fluorescence microscopy were indicative of abnormal accumulation of free cholesterol into siSMN-transfected fibroblasts, compared to siControl-transfected cells. This event was exacerbated following ATP-depletion/recovery treatment (figure 3C, stimulated), suggesting that, in activity-dependent context, SMN deficiency could affect the remodelling of cholesterol-enriched membrane domains. We also verify this issue in primary fibroblasts derived from a SMA type I patient. By a semiquantitative RT-PCR we showed that, despite the almost unchanged abundance of ABCA1 transcript in whole cell extracts, SMA type I fibroblasts exhibited a clear reduction of plasma membrane-associated ABCA1 transcript (figure 4A; figure 4B). These findings were in line with the results obtained in siSMN-transfected fibroblasts. Next, we monitored free cholesterol distribution by filipin staining (figure 4C, unstimulated). In SMA type I fibroblasts, we found an abnormal cholesterol accumulation compared to primary fibroblasts from a healthy individual. Moreover, this event appeared more evident following ATP-depletion/recovery stimulation (figure 4C, stimulated). Together, these findings provide a first demonstration of a correlation between SMN and cholesterol efflux regulators. Importantly, we showed that SMN not only associates to ABCA1 mRNA, but also affects its subcellular distribution.

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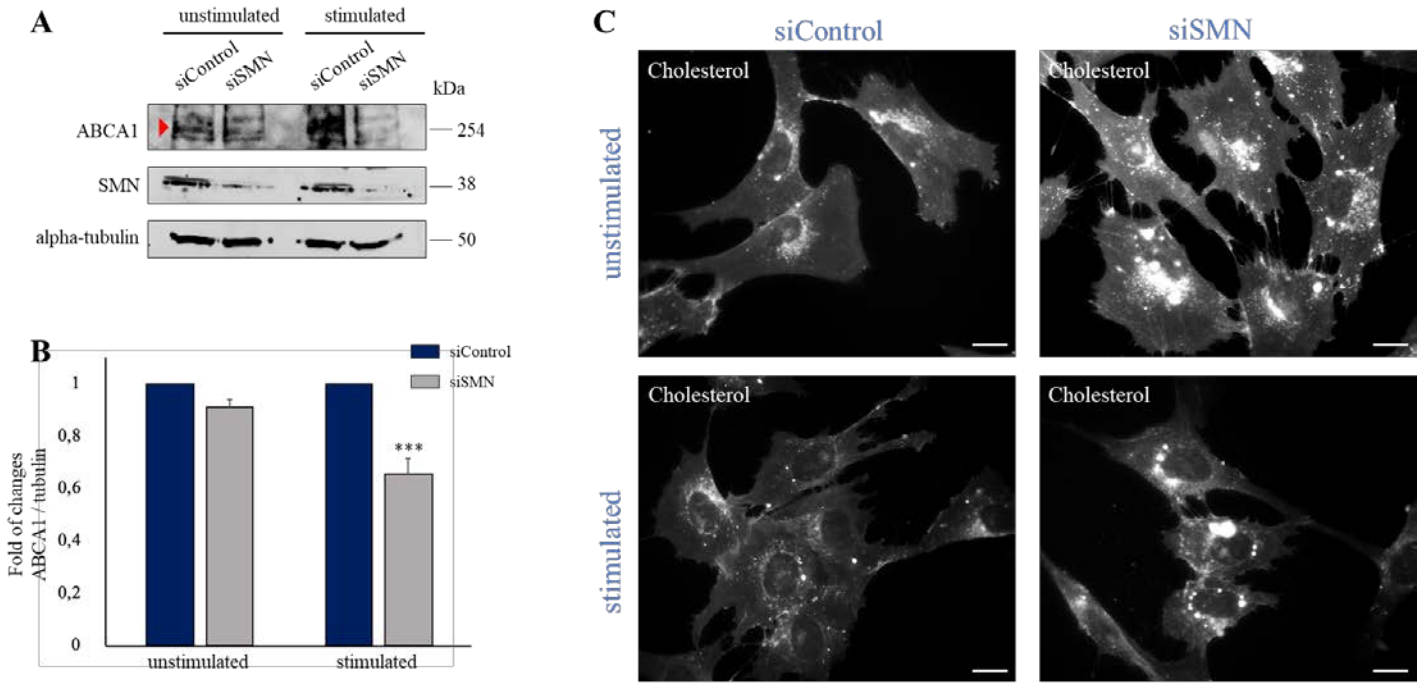


**Figure 3. SMN deficiency downregulates ABCA1 protein levels and causes intracellular accumulation of cholesterol.** (A) Representative western blot analysis of protein extracts from unstimulated and stimulated transfected cells. Equal amounts of proteins were blotted and checked for ABCA1 and SMN. Levels of alpha-tubulin were monitored as a control of protein loading. (B) Densitometric analysis of ABCA1 normalized to alpha-tubulin in unstimulated and stimulated siSMN transfected fibroblasts compared with the control (siControl). The graph illustrates the mean of three independent experiments. Error bars represent s.d.. Asterisks indicate significative differences using unpaired t-test (*** $P < 0.01$). (C) Representative images of free cholesterol in unstimulated and stimulated transfected human fibroblasts by filipin staining. Scale bars, 10 μm.

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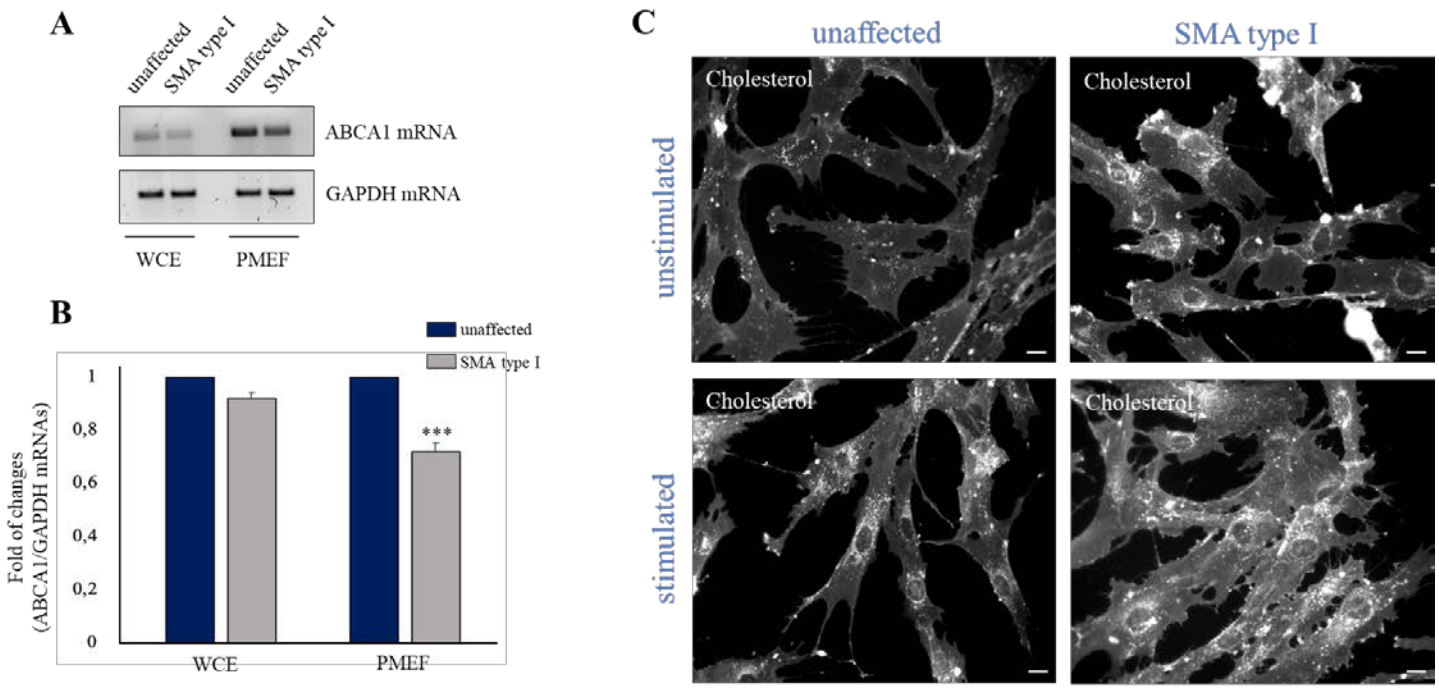


**Figure 4. ABCA1 mRNA localization and intracellular distribution of cholesterol are affected in SMA type I fibroblasts.** (A) ABCA1 mRNA and GAPDH mRNA were checked in WCE and PMEF of unaffected and SMA type I affected fibroblasts by semiquantitative RT-PCR. Panels are representative of three independent experiments. (B) Densitometric analysis of ABCA1 mRNA normalized to GAPDH mRNA in WCE and PMEF of unaffected and SMA type I affected fibroblasts. The graph illustrates the mean of three independent experiments. Error bars represent s.d.. Asterisks indicate significative differences using unpaired t-test (*** $P < 0.01$). (C) Representative images of free cholesterol in unstimulated and stimulated SMA type I fibroblasts compared to primary fibroblasts from a healthy individual by filipin staining. Scale bars, 10 μm.

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## 3. Discussion

SMA is a rare genetic disease whose complexity emerges by its pathological impact involving different organs, beyond neuromuscular system. It has been reported that SMA patients exhibit an increased risk of dyslipidemia, suggesting an implication of SMN in lipid metabolism [12]. Although intriguing, this aspect of SMA pathophysiology remains to be addressed.

We recently showed a relationship between SMN and plasma membrane-related networks [9, 10]. SMN not only associates to plasma membrane proteins, but also makes the PM competent to restrict protein synthesis at the subcellular level. Moreover, experimental evidence corroborates the concept that SMN may impact the membrane trafficking and endocytosis pathways [3]. These findings suggest that SMN can promote the establishment of specialized subdomains by a fine synchronization of membrane remodelling and protein synthesis control. This implies that the amount of SMN protein that a cell needs for proper performance depend on the extension of its endomembrane system. As known, SMN binds ribosomal proteins as well as their coding-transcripts [9, 10, 31]. Recently, Lauria and colleagues reported that SMN deficiency causes ribosome depletion at the beginning of the coding sequence of distinct mRNAs. Some of these SMN-specific mRNAs are linked to lipid metabolism, such as SREBF1 transcript [31]. Notably, SREBF1 is a transcription factor that targets the promoter sequence of genes involved in cholesterol biosynthesis and lipid homeostasis [32]. In this framework, we wanted to establish whether SMN can impact on lipid components of the plasma membrane. Since cholesterol is an essential structural constituent of the PM, we started to explore a relationship between SMN and ABCA1, a key regulator of cholesterol homeostasis. In human fibroblasts, we showed the existence of intracellular complexes in which SMN protein coexists with ABCA1 transcript. Indeed, a co-traffic of SMN protein and ABCA1 mRNA was also suggested by our imaging studies. As indicated by both biochemical and microscopy approaches, a pool of ABCA1 transcripts was prone to localize at the cell periphery and to associate with the PM. Importantly, membrane compartmentalization of ABCA1 mRNA was impaired by SMN deficiency, despite an overall increment of ABCA1 mRNA levels. Accordingly, ABCA1 protein, whose expression levels were increased in an activity-dependent context, appeared downregulated in SMN-depleted stimulated cells. One of downstream effects of the reduced expression levels of ABCA1 is the cytoplasmatic accumulation of cholesterol due to its efflux impairment [21]. Accordingly, in our system we demonstrated an aberrant distribution of cholesterol in siSMN-transfected fibroblasts and, most importantly, in SMA type I patient-derived fibroblasts. Thus, in a SMN deficiency background, the intracellular content of cholesterol appears dysregulated.

Noteworthy, ABCA1, in addition to regulating cholesterol efflux, modulates annexin A1 (ANXA1), which is associated with anti-inflammatory responses [33, 34]. Interestingly, an important paralog of ANXA1 gene is ANXA2, whose transcript displays an SMN-dependent axonal localization [35]. An interesting notion is that miR-183 levels are increased in SMN-deficient neurons [36]. Remarkable, it has been demonstrated that miR-183 binds the 3'UTR of ABCA1 mRNA and negatively regulates its expression [37, 38]. Collectively, these findings fit very well with the idea that SMN supports membrane dynamics by a fine-tuning of membrane-related factors. Homeostatic regulation of cholesterol is needed to remodel membrane platforms underlying specialized cellular activities. Notably, cholesterol biosynthesis and efflux are dysregulated in Amyotrophic Lateral Sclerosis (ALS) [39], a neuromuscular pathology sharing several clinical aspects with SMA. It is important to mention that cholesterol stabilizes neuromuscular junctions (NMJs) promoting their maturation from patch- to pretzel-type morphology [40]. Moreover, it has been reported that proper cholesterol content ensures a fine neuromuscular transmission and synaptic integrity [16]. This is a crucial issue in SMN-related networks, since NMJs dysfunction is an early event in SMA pathophysiology [41]. In this context, it is important to point out that cholesterol is essential for membrane expansion of glial cells, and it is one of the main lipid molecules in myelin. Notably, in a mouse model of SMA it has been shown that

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myelination defects as well as NMJ alterations may be reversed by a selective restoration of SMN protein levels in myelinating Schwann cells [42]. Interestingly, ANXA1 can trigger Schwann proliferation and migration following peripheral nerve injury [43]. Given the relationship between ANXA1 and ABCA1, it is plausible to suppose a critical role of ABCA1 also in Schwann cells activity. Indeed, in Tangier families a demyelinating multineuropathy condition has been described [44, 45]. These findings highlight an intriguing interplay between SMN and ABCA1 networks. So, it will be interesting to deeply assess whether and how SMN influences cholesterol homeostasis and its related pathways.

In conclusion, in the era of SMA-modifying therapies [46], this study provides a further understanding of molecular landscape, related to SMN functions, and can help to develop complementary approaches providing more clinical benefits for patients.

## 4. Materials and Methods

*Antibodies and Reagents*

The following antibodies were used: anti-SMN mouse monoclonal antibody (cat. no. 610647, BD Transduction Laboratories; work dilution for western blotting, 1:10,000; for immunofluorescence, 1:150); anti-ABC1 mouse monoclonal antibody (cat. no. sc-53482 Santa Cruz Biotechnology; work dilution for western blotting, 1:200); anti-alpha-tubulin mouse monoclonal antibody (cat. no. T6074, Sigma-Aldrich, St. Louis, MO, USA; work dilution for Western blotting, 1:2000). The secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories and used at a dilution of 1:5000. The Alexa Fluor488-conjugated secondary antibodies were purchased from Thermo Fisher and were used at a dilution of 1:200. Filipin (cat. no. F-9765) were purchased from Sigma-Aldrich.

*Cell cultures and transfection*

hTert-immortalized human fibroblasts, were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with heat inactivated 10% FBS (Australian, Gibco), penicillin-streptomycin (Gibco) and GlutaMAX (Gibco). Human fibroblasts from SMA type I patient (GM00232) and healthy control (GM08333) were obtained from Coriell Institute for Medical Research (Camden, NJ, USA), and grown in DMEM medium supplemented with 10% FBS, penicillin/streptomycin, and GlutaMAX. All cell cultures were maintained at 37 ◦C in a humidified atmosphere of 5% CO2. For knockdown experiments, cells were transfected with Lipofectamine 2000 (Thermo Fisher Scientific) and a combination of three siRNA-27 duplexes targeting the human SMN1 gene (OriGene), following manufacturer's instructions. Universal scrambled siRNA duplex was used as negative control. Cells were harvested after 48 h post transfection.

*ATP depletion and recovery assay*

The ATP depletion/recovery assay was performed as described previously [9, 29]. Briefly, fibroblasts were incubated in PBS supplemented with 1 mM CaCl2, 1 mM MgCl2 and 20 mM NaN3, for 1 h. NaN3-containing buffer was then replaced with fresh medium supplemented with heat inactivated 10% FBS (Australian, Gibco), for 30 min, allowing ATP recovery.

*Preparation of plasma membrane-enriched fractions*

Plasma-membrane-enriched fractions (PMEFs) was isolated as previously described [9]. Briefly, cells were lysed in buffer A (5 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM DTT and 320 mM sucrose). Extracts were passed through a 26G needle five times and centrifuged at 1000 g for 10 min at 4°C. The supernatant was kept, and the pellet was quickly vortexed in the presence of original volume of lysis buffer and centrifuged at 1000 g for 10 min at 4°C. The two supernatants were pooled and centrifuged at 24,000 g for 20 min at 4°C in a Beckman SW41 rotor. The supernatant was discarded, and the pellet was resuspended in 12 ml of buffer B (5 mM Tris-HCl pH 7.4, 1 mM EGTA and 1 mM DTT), and

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centrifuged at 24,000 g for 30 min at 4°C in a Beckman SW41 rotor. The supernatant was discarded. The pellet was aliquoted and processed RNA extractions.

*Immunofluorescence microscopy*

Cells were fixed with 4% formaldehyde in PBS, permeabilized in 0.2% Nonidet P40 (Boehringer Mannheim) for 20 min and blocked with 1% BSA in PBS at room temperature. Samples were incubated sequentially with the appropriate primary and secondary antibodies. Slides were mounted with ProLong with Dapi (Thermo Fisher Scientific) and examined by conventional epifluorescence microscope (Olympus BX53; Milano, Italy). For free cholesterol staining by Filipin, cells were fixed in 4% formaldehyde in PBS, incubated with 1.5 mg glycine/ml PBS for 10 min at room temperature to quench the formaldehyde and stained with 0.05 mg/ml in PBS filipin for two hours at room temperature. Slides were mounted with ProLong (Thermo Fisher Scientific) and examined by conventional epifluorescence microscope (Olympus BX53; Milano, Italy), using excitation at 340-380 nm and emission at 385-470 nm. Images were captured by a SPOT RT3 camera and elaborated by IAS software.

*RNA Immunoprecipitation (RIP) assay*

Cells were resuspended in IP Buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM NaVO4, 0.1% Triton X-100, 5% glycerol and complete protease inhibitor cocktail (Roche)), in the presence of RNase inhibitors (Thermo Fisher Scientific). Extracts were vortexed 3 times for 10 seconds, incubated in ice for 20 minutes and centrifuged at 10,000 g for 7 min at 4°C. For the immunoprecipitation assay, the protein lysate was pre-cleared with Protein A/G-Agarose beads (Roche, Indianapolis, IN, USA), pre-saturated in 2% BSA-PBS, by replacing beads 3 time within 90 minutes, at 4 °C. Then 750 μg of extract was immunoprecipitated in IP buffer overnight with the anti-SMN monoclonal antibody. As negative control, the immunoprecipitation was carried out with mouse IgG-beads. The beads were washed five times for 5 min at 4 °C in IP buffer and once in PBS buffer. The immunoprecipitated samples were resuspended in IP buffer. A portion of immunoprecipitation was analyzed by western blot analysis. RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. RNAs were converted to cDNAs using a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific).

*Western blot analysis*

All samples were processed in sample buffer and incubated at 100° C for 10 minutes, except samples designated for ABCA1 detection. For ABCA1 detection, samples were incubated with sample buffer containing beta-mercaptoethanol at room temperature for 30 minutes. Protein extracts were electrophoresed through standard 6% SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare; Milano, Italy). Immunodetection of the reactive bands was revealed by chemiluminescence (ECL kit, GE Healthcare), and analyzed by iBright 1500 (Thermo Fisher Scientific Inc.).

*RNA extraction, retrotranscription and semiquantitative real-time PCR (RT-PCR)*

RNA from whole cell extract and PMEF fraction was extracted using TRIzol® reagent according to the manufacturer's instructions and was then reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). A semiquantitative PCR (RT-PCR) assay was performed in triplicate using the BioMix 2X (Bioline) according to the manufacturer's instructions. Primer sequences used in this study are shown in Table 1.

*Padlock Assay*

Phosphorylation of the padlock probe and Padlock assay were performed as previously described [9].

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*Quantification and Statistical Analysis*

All experiments were performed on at least three independent biological replicates. Data are presented as mean ± s.d. Statistical analysis was performed using the GraphPad Prism software. Data were analyzed using an unpaired t-test; $p < 0.01$ was considered statistically significant.

**Table 1. Oligos used in the study**

| **Primer name** | **Primer sequence (5'-3')** |
|---|---|
| RT-PCR GAPDH F | CATGAGAAGTATGACAACAGCCT |
| RT-PCR GAPDH R | AGTCCTTCCACGATACCAAAGT |
| RT-PCR POLB F | GTGAGACAAAGTTCATGGGTGT |
| RT-PCR POLB R | GTGAAACCCTTTTCTAGGGCAT |
| RT-PCR ABCA1 F | TACATCTCCCTTCCCGAGCA |
| RT-PCR ABCA1 R | GGAGCTGGAGCTGTTCACAT |
| Padlock Probe ABCA1 | CATGTCACTCCAGCTTTTTTTTCTCAATTCTGCTACTTTACTACC TCAATTCTGCTACTGTACTACTTTTTTCATCACCTCCTGTCG |
| RCA Primer | AGTACAGTAGCAGAATTGAG |
| AlexaFluor 595-labelled probe | CTCAATTCTGCTACTTTACTAC |

**Supplementary Materials:** Not applicable.

**Author Contributions:** FG: investigation; formal analysis; writing-original draft; AO, CP (Cinzia Pisani), MF, GF: formal analysis; data curation; AC, MdV: resources, review & editing; MC, MI, NC: investigation, writing - review & editing; CP (Claudio Passananti): conceptualization; supervision; funding acquisition; writing-original draft; MGDC: supervision; conceptualization; investigation; writing—original draft preparation. All authors were involved in writing the paper and had final approval of the submitted version.

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