*Original Article*
# Prognostic significance of alpha-2-macrglobulin and low-density lipoprotein receptor-related protein-1 in various cancers

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**Abstract:** Cancer is the leading cause of death worldwide. The World Health Organization (WHO) estimates that 10 million fatalities occurred in 2023. Breast cancer (BC) ranked first among malignancies with 2.26 million cases, lung cancer (LC) second with 2.21 million cases, and colon and rectum cancers (CC, CRC) third with 1.93 million cases. These results highlight the importance of investigating novel cancer prognoses and anti-cancer markers. In this study, we investigated the potential effects of alpha-2 macroglobulin and its receptor, LRP1, on the outcomes of breast, lung, and colorectal malignancies. Immunohistochemical staining was used to analyze the expression patterns of A2M and LRP1 in 545 cases of invasive ductal breast carcinoma (IDC) and 51 cases of mastopathies/fibrocystic breast disease (FBD); 256 cases of non-small cell lung carcinomas (NSCLCs) and 45 cases of non-malignant lung tissue (NMLT); and 108 cases of CRC and 25 cases of non-malignant colorectal tissue (NMCT). A2M and LRP1 expression levels were also investigated in breast (MCF-7, BT-474, SK-BR-3, T47D, MDA-MB-231, and MDA-MB-231/BO2), lung (NCI-H1703, NCI-H522, and A549), and colon (LS 180, Caco-2, HT-29, and LoVo) cancer cell lines. Based on our findings, A2M and LRP1 exhibited various expression patterns in the examined malignancies, which were related to one another. Additionally, the stroma of lung and colorectal cancer has increased levels of A2M/LRP1 areas, which explains the significance of the stroma in the development and maintenance of tumor homeostasis. A2M expression was shown to be downregulated in all types of malignancies under study and was positively linked with an increase in cell line aggressiveness. Although more invasive cells had higher levels of A2M expression, an IHC analysis showed the opposite results. This might be because exogenous alpha-2-macroglobulin is present, which has an inhibitory effect on several cancerous enzymes and receptor-dependent signaling pathways. Additionally, siRNA-induced suppression of the transcripts for A2M and LRPP1 revealed their connection, which provides fresh information on the function of the LRP1 receptor in A2M recurrence in cancer. Further studies on different forms of cancer may corroborate the fact that both A2M and LRP1 have high potential as innovative therapeutic agents.

Keywords: Breast cancer, IDC, lung cancer, NSCLC, colorectal cancer, CC, alpha-2-macroglobulin, A2M, low-density lipoprotein receptor-related protein-1, LRP1

## Introduction

Cancer is caused by many changes in various genes. All cancers are very different from one another at the molecular level, but some have the same mutations. Most malignancies originate from an accumulation of mutations that result in morphological and phenotypic alterations. These changes in DNA may result from environmental, biological, or chemical factors. Normal cell genes involved in homeostasis that regulate the cell cycle, proliferation, and apop-

tosis must change for cancer to form. This causes the activation of oncogenes, anti-apoptotic and pro-proliferating genes, and the inhibition of tumor suppressor genes. After eluding the typical cell cycle and evading programmed cell death, cancer cells must overcome the barrier of replicative senescence to achieve immortality. Establishing effective nutrition and oxygen uptake by cancer cells is the last phase of their growth. This step is necessary to support high levels of cellular metabolic and proliferative activity. Scientists from all around the world are working to pinpoint certain key elements and indicators that could serve as a common denominator of cancer development to better understand the molecular mechanisms underlying the genetic modifications responsible for carcinogenesis.

One of the most promising approaches in cancer biology is pan-cancer analysis. The major goal of pan-cancer research is to identify commonly altered genes and the molecular mechanisms that are shared by a variety of cancer forms, regardless of tumorigenesis. As a result, a variety of potent technologies are being utilized alongside more conventional technologies, including next-generation sequencing (NGS); pyrosequencing; and RNA, miRNA, and proteome sequencing. Greater cancer detection and therapy are made possible by greater knowledge of the real nature of cancer mutations and their effects. Therefore, it is crucial to identify novel potential cancer markers.

Tumors are often categorized based on the organ or tissue from which they originate, their specific kind, the WHO grading system, and their dissemination based on the tumor-node-metastasis (TNM) staging system. There are many different types of cancer that can appear anywhere in the body and at practically any age. Hematologic (blood) malignancies and solid tumor cancers are the two main categories of tumors. They all share many characteristics, but their main differences lie in how they develop, proliferate, disseminate, and react to therapy. Although different forms of cancer vary, they differ from those of normal cells. For instance, cancer cells can proliferate even in the absence of signaling molecules (growth factors), avoid signals that would otherwise cause them to stop and undergo apoptosis, invade nearby tissues, start angiogenesis to obtain sufficient nutrients and oxygen, evade the immune system, and coerce immune cells to support their survival in an organism. Therefore, the most desirable cancer indicators have a strong relationship with the characteristics listed above. Targeting markers that are unique to cancer and not to normal cells will result in a greater response to therapy with a lower likelihood of adverse effects in practice. Globally, scientists are working hard to develop treatments that specifically target the aberrant characteristics of tumor cells.

It is estimated that approximately 43% of all cancer-specific genetic aberrations are shared by most cancer types [1-3]. This is a very high number that supports a modern approach that focuses on multiple cancer-associated markers. Modern anti-cancer treatments are based on the origin of the cancer, which sometimes prevents similarities between various types of malignancies. The molecular and clinical significance of multiple cancer-associated markers is still uncertain; therefore, it is important to provide more experimental data on the mutational landscapes of different cancers. Various cancers share the same risk factors, both genetic and environmental for example, mutations in genes such as *BRCA1* (Breast Cancer Type 1 Susceptibility Protein), *EGFR1* (Epidermal Growth Factor Receptor 1), *TP53* (Tumor Protein P53), *KRAS* (KRAS Proto-Oncogene, GTPase), *TERT* (Telomerase Reverse Transcriptase)*,* and *MYC* (Myc Proto-Oncogene) [4-9]. Of the almost 30,000 genes present in the human genome, only a few are associated with cancer progression; however, this number is sufficient for cancer cells to change the hemostasis of the whole body. As mentioned above, many cancer-specific mutations are typical of cancerous cells and not present in healthy cells. Therefore, it is important to define those that are shared by the largest number of cancer types. In this study, we aimed to determine whether alpha-2-macroglobulin (A2M) and its receptor LRP1 (CD91, A2MR) can be considered as multiple cancer-associated markers in breast, lung, and colorectal cancers.

*A2M and LRP1 in homeostasis*

Alpha-2-macroglobulin (A2M), also known as ovostatin, is a large plasma glycoprotein that acts as a proteinase inhibitor. It was first described in 1946 [10]. In humans, *A2M* is located on chromosome 12p13.31, which

encodes a functional homotetramer (720 kDa) linked to 180 kDa subunits composed of 1474 amino acid residues. A2M belongs to the thioester-containing protein (TEP) family. It undergoes proteolytic processing as part of its role in immune defense mechanisms. All of the members of the TEP family have been classified as belonging to the alpha-2-macroglobulin family, including A2M, PZP (Pregnancy Zone Protein), $\alpha_1$I3, CPAMD8 (C3- and PZP-like alpha-2-macroglobulin domain-containing protein 8), and CD109 (C3- and PZP-like alpha-2-macroglobulin domain-containing protein 7), as well as their C3 subunit structural "lookalikes" C4 and C5 [11-15]. The most recognized and highly conserved motif of A2M forms a hyperactive thioester bond (C-X-E-Q; X for L or G) [16-19].

Although the expression of macroglobulin is strongly linked to liver tissue, it has also been discovered to be released by the reproductive tract, the brain, and the heart [20-23]. The most important functions in maintaining homeostasis are to actively bind and transport cytokines, hormones, apolipoproteins, growth factors, and misfolded proteins to suppress enzyme cascades and the kallikrein-kinin system, regulate blood coagulation processes and fibrinolysis, and initiate inflammatory reactions [22, 24]. In blood and cerebral fluid, A2M levels oscillate between 1.5 and 2 mg mL$^{-1}$ and 1.0 and 3.6 μg mL$^{-1}$, respectively [17, 25]. The presence of A2M in the blood at this concentration is driven by the facilitation of access to all parts of the body. Because of the presence of a bait region containing a large number of proteinase cleavage sites, A2M appears to be a very common protein partner for a wide range of diverse proteases. A2M bait region cleavage results in the covalent deceiving of proteins within a steric half-cage [26]. This mechanism requires conformational changes, resulting in compact tetrameric structure formation and unveiling the binding site for low-density lipoprotein receptor-related protein-1 (LRP1) [12, 18, 21, 27].

LRP1 (CD91) binds to the C-terminal RBD domain region of alpha-2-macroglobulin after induction by peptidases. The low-density lipoprotein receptor-related protein is a 600 kDa membrane-bound receptor and an important member of the LDL receptor family, including the apolipoprotein E receptor 2/LRP8, megalin/LRP2, and the VLDL receptor. It has been proven that all LDL receptors are characterized by the ability to overlap each other's functions under specific physiological circumstances [28]. CD91 is expressed in various types of cells as a single-chain precursor that, during post-translational maturation, is arranged into a 515 kDa α-chain and an 85 kDa transmembrane β-chain that are covalently bound together [29, 30]. The α-chain of CD91 is formed of four clusters of 2, 8, 10, and 11 complement-like repeats (CRs) limited by β-propeller modules and epidermal growth factor-type repeats [31]. It has been proven that A2M binds to LRP1 with a very high affinity, mostly via cluster 2 containing eight CRs, where hydrophobic interactions between RAP and CR complexes are most crucial. The RBD region of macroglobulin exposes lysines K1393 and K1397, revealing a conserved ΨKXΨK motif, where Ψ is hydrophobic and X is an amino acid residue [32]. The dissociation of ligand-receptor complexes is essential for receptor recycling and function. In a neutral pH environment, ligands actively interact with the LRP1 binding site and are then released into the endosomes at pH 6.0.

It has been proven that LRP1 subcellular localization is highly dependent on the presence of lipid rafts. Nevertheless, the receptor can migrate into the clathrin-coated regions of the plasma membrane, where it can be successfully endocytosed [33-35]. Additionally, CD91's ligands can undergo transcytosis in endothelial cells [36, 37]. The first LRP1 ligand is apolipoprotein E [38]. Then, LRP1 was proven to be an effector receptor for activated A2M [39, 40]. Diverse proteases, protease inhibitors, viral proteins, and toxins have been identified as CD91 ligands [28]. There is growing evidence for abundant interactions and the role of LRP1 in maintaining homeostasis. It can interact with diverse plasma membrane proteins, such as Plxdc 1/TEM-7, whose functions are associated with angiogenesis, Fe65-promoting APP endocytosis, N-methyl-D-aspartate the PSD-95 co-ligand, and the urokinase receptor uPAR [41-47]. Moreover, CD91 functions are associated with antigen presentation and stimulation of CD8$^+$ T cells, mostly through the mediation of heat shock proteins (HSPs) [48].

Additionally, LRP1 expression levels increase during hypoxia, which is typical for tumor formation. Both alpha-2-macroglobulin and LRP1 are associated with inflammatory processes, not

only in the context of injuries, infections, or arteriosclerosis, but also in the context of cancer progression. Therefore, further investigation of their role in tumorigenesis is warranted.

*Role of A2M and LRP1 in cancer progression*

For decades, proteases have been associated with tumor progression, invasion, and metastasis, mostly because of their ability to degrade the extracellular matrix. Recent studies have demonstrated that proteases are involved in the early stages of cancer initiation and progression, especially in cancer cell growth. Cancer cells show incredible abilities to interact with and influence not only other cancer cells but also stromal cells and the extracellular matrix by controlling protease activity. One of the possibilities for protease control is the use of specific inhibitors. A major proteinase inhibitor in mammals is A2M, which can irreversibly inactivate a wide spectrum of proteins, regardless of their catalytic mechanism and substrate specificity. High plasma levels of A2M are unquestionable; nevertheless, they differ in various types of cancer [49-53].

Unfortunately, there is a small amount of knowledge regarding the expression patterns and role of A2M in cancer progression, limited to prostate and bladder cancers, breast cancer, leukemia, and astrocytoma. Researchers have demonstrated the potential role of A2M in prostate cancer patients, where it can interact with the serine protease prostate-specific antigen (PSA) [54]. In prostate cancer patients, A2M plasma levels can be elevated even above 4.4 μg/mL, simultaneously forming PSA-A2M complexes. Moreover, A2M plasma levels decrease with tumor grade. This could be due to the fact that the overproduction and oversecretion of PSA overwhelm the production of macroglobulin inhibitors by the liver, or even because prostate cancer cells produce factors that affect A2M levels. Additionally, a statistically significant correlation was observed between the increasing levels of A2M and IL-6 (interleukin 6), which can be explained by alpha-2-macroglobulin's carrier role. Artificially decreasing A2M levels resulted in a decrease in TGF-β1 (transforming growth factor-beta 1) levels and altered cancer cell growth kinetics. PSA-A2M serum activity can be actively suppressed by peptide-based boronic acid inhibitors of PSA [55]. Additionally, it has been proven that A2M

binds to prostate cancer cells via direct interaction with immunoglobulin protein GRP78 (Heat Shock 70 kDa Protein 5) and activates the insulin response [56]. A2M acts as a growth factor, increasing the proliferation rate. Therefore, constructing an antibody against GRP78 may be a successful tool in modern therapy for prostate cancer. A2M can not only interact with the LRP1 receptor on T cells but can also be expressed on the B cell surface, although the true nature of this observation requires further investigation.

A2M has been identified as an antigen-causing aggregation of IgG in lymphocytic leukemia. These IgG-antigen aggregates occur on the surface of malignant B cells as well as on free detached complexes.

Analysis of plasma samples from bladder cancer patients revealed that, among other protein markers, A2M levels differed from those observed in the control group. Similar to prostate cancer patients, alpha-2-macroglobulin levels were also significantly increased in our study, which can be used in future clinical practice [57].

It has been proven that AS1-A2M, the antisense A2M, is upregulated in breast cancer patients compared to control samples. AS1-A2M is highly associated with ER-negative breast cancer, resulting in worse progression-free and metastasis-free survival [52, 53]. AS1-A2M successfully enhanced breast cancer cell proliferation, migration, and invasion-regulating cell adhesion molecule pathways, such as CD2, CD8A, and selectin L (SELL), which can serve as a potential prognostic marker of BC.

In astrocytomas, the transformation-associated isoform A2M (A2M*) interacts with LRP1 to target cancer cell growth, invasion, and migration. Interestingly, native A2M does not show affinity for LRP1, suggesting that only A2M* has strong tumor-suppressive features [58]. It can also effectively bind to GRP78, resulting in the inhibition of cell proliferation and the creation of an antiapoptotic milieu [59, 60]. It has been postulated that the A2M*/LRP1 axis may regulate cancer cell growth by interfering with the Wnt/β-catenin signaling pathway. Overexpression of LRP1 in astrocytoma cells opens a path for A2M* to influence Wnt/β-catenin signaling by relocating β-catenin to the plasma

membrane and further upregulating LRP1 and FZD [58]. Moreover, decreased plasma levels of A2M* at higher ages may be linked to carcinogenesis.

Compared to alpha-2-macrogolublin, LRP1's influence on tumor growth and invasiveness is well acknowledged [61]. This could be caused mainly by the fact that LRP1 can interact not only with A2M but also with various biological ligands, such as viruses, growth factors, lipoproteins, matrix compounds, and proteinase inhibitors. Specific LRP1 membrane-anchored complexes have been shown to be associated with the aggressiveness of various cancer types [62-66]. Interestingly, there is no shortage of opposite observations, where exogenously added LRP1 antagonist RAP resulted in decreased invasiveness of breast cancer cells [67-69]. Moreover, the abolition of LRP1 expression in human thyroid carcinoma leads to increased MMP-2 (Matrix Metallopeptidase 2) and uPA (Urokinase-Type Plasminogen Activator) levels in the extracellular space. LRP1 promotes the mediated endocytic uptake of TIMP-2-free pro-MMP-2 [70-72]. Therefore, silencing LRP1 induces a reduction in tumor cell migration and invasiveness.

Most published data indicate the procancerous abilities of LRP1. For example, an increase in the T allele of C766T in *LRP1* is responsible for an increased risk of breast cancer [73]. The same overexpression was observed in high-grade astrocytomas [74]. Increased levels of *LRP1* mRNA and protein are observed in the majority of endometrial carcinomas, suggesting its role in endometrial cancer formation [75, 76]. Similar to A2M, LRP1 has been shown to regulate MMP-2 and Matrix Metallopeptidase 9 (MMP-9), promoting cell migration and invasion in glioblastoma [77]. Knockdown of *LRP1* in the U87 cell line resulted in decreased migration and invasiveness of cancer cells. This could occur via the ERK-dependent signaling pathway [77-79]. Another type of LRP1 involvement in cancer cell physiology is the promotion of cell invasion through ERK (Mitogen-Activated Protein Kinase 1) activation and inhibition of the JNK (Mitogen-Activated Protein Kinase 8) signaling pathway [80]. This could be due to the direct binding of LRP1 to PDGFR-β in endosomes [81, 82]. In glioblastoma multiforme, LRP1 forms signaling complexes with eHsp90, regulating the activation of EphA2 receptor tyrosine kinase, thereby simulating cell migration and invasion [83].

In addition to the pro-invasive and pro-proliferative abilities of LRP1, it has also been proven that this LDL receptor is involved in apoptosis. Successful knockdown of LRP1 resulted in a significant decrease in Caspase-3 levels, IR signaling, AKT (AKT Serine/Threonine Kinase 1) phosphorylation, and induction of apoptosis [84]. LRP1 can activate A2M by direct binding, leading to the activation of the AKT signaling pathway in Schwann cells [82]. However, the precise nature of these mechanisms remains unclear.

Current knowledge regarding the role of A2M and the CD91 receptors in cancer progression is still very limited. Therefore, in this study, we demonstrate for the first time the expression patterns and correlations of A2M and LRP1 in breast, lung, and colorectal cancers.

## Materials and methods

### Patients and clinical samples

The presented experiments were conducted using archival paraffin blocks, as follows:

● Invasive ductal breast carcinomas (n = 545) and fibrocystic breast disease (FBD) (n = 51) were obtained during surgical resection in 2010-2016 at the Polish Mother's Memorial Hospital Research Institute in Lodz.

● Non-small cell lung carcinomas (n = 256) and non-malignant lung tissue (NMLT) (n = 45) were obtained during surgical resection between 2007 and 2019 at the Lower Silesian Center of Lung Diseases in Wroclaw.

● Colorectal carcinomas (n = 108) and non-cancerous colorectal tissue (NMCT) (n = 25) were obtained from patients treated at the Lower Silesian Oncology Center in Wroclaw, Poland, from 2013 to 2018.

Paraffin sections of the cancerous samples were stained with hematoxylin and eosin (H&E) to verify the appropriateness of the immunohistochemical (IHC) analyses. Clinical data were derived from hospital archives and are summarized in **Tables 1-3**.

**Table 1.** Clinicopathological data of the studied cases of invasive ductal breast carcinoma

| Characteristic | No. (%) of patients (n = 495) |
|---|---|
| *Tumor size* | |
| T1 (<2 cm) | 190 (38.0) |
| T2 (2-5 cm) | 129 (26.0) |
| T3 (>5 cm) | 130 (26.0) |
| T4 | 46 (9.0) |
| *Tumor stage - pT* | |
| 1 | 313 (63.23) |
| 2 | 152 (30.71) |
| 3 | 19 (3.84) |
| 4 | 11 (2.22) |
| *Grading* | |
| I | 92 (18.59) |
| II | 312 (63.03) |
| III | 91 (18.38) |
| *Nodal status - pN* | |
| N0 | 298 (60.20) |
| N1 | 167 (33.74) |
| N2 | 18 (3.64) |
| N3 | 12 (2.42) |
| *ER status* | |
| Positive | 324 (65.45) |
| Negative | 171 (34.55) |
| *PR status* | |
| Positive | 312 (63.03) |
| Negative | 183 (36.97) |
| *HER2 status* | |
| Positive | 311 (62.83) |
| Negative | 184 (37.17) |
| *Triple-negative status* | |
| Positive | 40 (8.08) |
| Negative | 455 (91.92) |

**Table 2.** Clinicopathological data of the studied cases of non-small cell lung colon cancer

| Characteristic | No. (%) of patients (n = 256) |
|---|---|
| *Age (years)* | |
| Mean | 67 ± 7.64 |
| Range | 45-84 |
| *Gender* | |
| Male | 150 (59.0) |
| Female | 106 (41.0) |
| *Histology* | |
| Adenocarcinoma | 116 (45.0) |
| Squamous | 140 (55.0) |
| *Tumor size* | |
| T1 (<2 cm) | 61 (23.83) |
| T2 (2-5 cm) | 121 (47.27) |
| T3 (>5 cm) | 46 (17.97) |
| T4 | 28 (10.94) |
| *Lymph nodes* | |
| N0 | 140 (54.69) |
| N1, N2, N3 | 116 (45.31) |
| *pTNM* | |
| 1A | 42 (16.41) |
| 1B | 49 (19.14) |
| 2A | 30 (11.72) |
| 2B | 29 (11.33) |
| 3A | 37 (14.45) |
| 3B | 42 (16.41) |
| 4 | 27 (10.56) |
| *Grade* | |
| G1 | 55 (21.48) |
| G2 | 130 (50.78) |
| G3 | 71 (27.73) |

*Histopathological analysis of IHC reactions*

The immunocytochemical expression of A2M and LRP1 was studied using the Immu-noReactive Score (IRS) according to Remmele and Stenger, which considers both the intensity of the color (staining) of the reaction and the percentage of positively stained cells (**Table 4**). The final score ranged from 0 to 12. Statistical analysis was performed using the Mann-Whitney U-test, the ANOVA Kruskal-Wallis test, and the Spearman test. Differences were considered statistically significant at $P<0.05$.

*Cell lines*

For our experiments, the following cell lines were used:

● Breast carcinoma cell lines MCF-7, T-47D, MDA-MB-231 (obtained from American Type Culture Collection ATCC, Manassas, VA, USA), SK-BR-3, BT-474 (from the Cell Lines Collection of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Science, Wroclaw, Poland), and MDA-MB-231/BO2 (courtesy of Dr. Philippe Clezardin, INSERM U664, France), as well as immortalized normal breast cells hTERT-HME1 (ME16C) from ATCC. The breast cancer cell lines were cultured in α-MEM medium sup-

**Table 3.** Clinicopathological data of the studied cases of invasive ductal breast carcinoma

| Characteristic | No. (%) of patients (n = 108) |
|---|---|
| *Age (years)* | |
| Mean | 68.65 ± 10.87 |
| Range | 42-91 |
| *Gender* | |
| Male | 69 (63.89) |
| Female | 39 (36.11) |
| *Location* | |
| Proximal colon | 49 (45.37) |
| Distal colon | 59 (54.63) |
| *Dukes'* | |
| A | 2 (1.85) |
| B1 | 20 (18.52) |
| B2 | 35 (32.41) |
| C1 | 5 (4.63) |
| C2 | 35 (32.41) |
| C3 | 3 (2.78) |
| D | 8 (7.41) |
| *Grade* | |
| G1 | 7 (6.48) |
| G2 | 81 (75.00) |
| G3 | 20 (18.52) |
| *Treatment* | |
| RT | |
| Yes | 64 (59.26) |
| No | 44 (40.74) |
| Adjuvant 5-FU-based chemotherapy | |
| Yes | 73 (67.59) |
| No | 35 (32.41) |

**Table 4.** Evaluation of immunohistochemical reactions with the use of the ImmunoReactive Score (IRS)

| % positive cells | Intensity of reaction | IRS |
|---|---|---|
| 0 = no positive cells | 0 = no color reaction | 0-1 = negative |
| 1 ≤10% positive cells | 1 = mild reaction | 2-3 = mild |
| 2 = 10-50% positive cells | 2 = moderate reaction | 4-8 = moderate |
| 3 = 51-80% positive cells | 3 = strong reaction | 9-12 = strong |
| 4 ≥81% positive cells | | |

plemented with 2 mM L-glutamine (Lonza, Basel, Switzerland), 10% fetal calf serum (FCS; Invitrogen Carlsbad, CA, USA), and antibiotics. The ME16C cell line was cultured in the MEGM Bulletkit medium (Lonza, Basel, Switzerland). All media were supplemented with FBS (Sigma) to a final concentration of 10%. The cell lines were cultured at 37°C in 5% $CO_2$.

● The lung cancer cell lines NCI-H1703, NCI-H522, and A549, together with the normal lung fibroblast cell line IMR-90 (obtained from American Type Culture Collection (ATCC)). The NCI-H1703 and NCI-H522 cell lines were cultured in RPMI-1640 growth medium with the addition of 2 mM L-glutamine. The A549 cell line was cultured in high-glucose DMEM with 2 mM L-glutamine, whereas the IMR-90 fibroblastic cell line was cultured in MEM. All media were supplemented with FBS (Sigma) to a final concentration of 10%. The cell lines were cultured at 37°C in 5% $CO_2$.

● Colorectal cell lines LS 180, Caco-2, HT-29, and LoVo were obtained from the American Type Culture Collection (ATCC). The LS 180 cell line was cultured in Eagle's Minimum Medium, and CaCo-2 with EMEM (Lonza, Basel, Switzerland) with 1% sodium propionate, 1% non-essential amino acids, and 20% FBS (all from Sigma-Aldrich®, St. Louis, MO, USA). The HT-29 cells were cultured in McCoy's 5A medium, and the LoVo cells were cultured in F12-K medium. The cell lines were cultured at 37°C and 5% $CO_2$. All the studied cell lines were arranged according to their aggressiveness potential, from the lowest to the highest.

*Immunohistochemistry (IHC)*

For IHC reactions, we used cancerous and non-cacerous tissue samples fixed in 10% buffered formalin and embedded in paraffin. Immunohistochemical analysis of A2M and LRP1 was performed using mouse monoclonal antibodies against A2M (1:200; 66126-I-Ig; ProteinTech, Rosemont, IL, USA) and LRP1 (SM17-29P; OriGene, Rockville, MD, USA). IHC was performed using Autostainer Link 48 (DakoCytomation, Glostrup, Denmark) to provide reliable and repeatable conditions.

*RNA extraction, cDNA synthesis, and real-time PCR reactions*

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and transcribed

to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. RT-qPCR was conducted in 20 µL volumes using the iTaq Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a 7500 real-time PCR system. The TaqMan-specific probes used in the experiment (Hs00929971_m1 for *A2M*, Hs00233856_m1 for *LRP1*, and Hs 99999903_m1 for *ACTB* as a reference gene) were also obtained from Applied Biosystems. All the above-mentioned reactions were performed in triplicate under the following conditions: activation of polymerase at 50°C for 2 min, initial denaturation at 94°C for 10 min followed by 40 cycles of denaturation at 94°C for 15 s, and annealing and elongation at 60°C for 1 min. The relative mRNA expression of the studied markers was calculated using the ΔΔCt method.

### SDS-PAGE and Western blotting

The cell lines were lysed on ice in Cell Lysis Buffer (Thermo Fisher Scientific, Waltham, MA, USA) with a cocktail of inhibitors (Sigma, St. Louis, MO, USA), 250 units of Benzonase® (Merck Millipore, Bedford, MA, USA), and 2 mM PMSF (phenylmethanesulfonyl fluoride). Lysates containing 30 µg of total proteins were combined with 4x SDS-PAGE gel-loading buffer (200 mM Tris-HCl, pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, and 40% glycerol), loaded onto 10% acrylamide gels, and separated by SDS-PAGE under reducing conditions before being transferred to PVDF membranes. Following the protein transfer, the membranes were incubated for 1 hour at room temperature in a blocker solution (4% BSA in TBST buffer), followed by an overnight incubation at 4°C with anti-A2M (1:400) and anti-LRP1 (1:500) antibodies. The membranes were then washed with TBST buffer and incubated for 1 h at room temperature with HRP-conjugated anti-rabbit and anti-mouse secondary antibodies, diluted 1:3000 (709-035-149 and 715035-150, respectively; Jackson ImmunoResearch, Mill Valley, CA, USA), then washed and treated with the Immun-Star HRP Chemiluminescence Kit (Bio-Rad). Rabbit anti-human β-actin monoclonal antibody (#4970; Cell Signaling Technology, Danvers, MA, USA), diluted 1:1000, was used as an internal control. Western blot results were analyzed using the ChemiDoc MP system (Bio-Rad).

### siRNA transfection

Ambion pre-designed siRNAs (*GAPDH* siRNA was used as a positive control and scrambled sequence siRNA as a negative control) were used in this study. The siRNAs used were s820 (siRNA1/A2M) and s821 (siRNA2/A2M) for A2M, and 106762 (siRNA1/LRP1) and 106763 (siRNA2/LRP1) for LRP1 (Thermo Fisher Scientific). MCF-7 and A549 cells were cultured in 6-well plates, as previously described. The concentration of siRNA and the amount of transfection reagent were measured experimentally. Before transfection, cells were trypsinized, spun at 1000 rpm for 5 min at 4°C, and resuspended in fresh medium. Ambion's siPORT NeoFX (4 l/well) lipid-based transfection reagent and siRNAs (50 nM final concentration) were independently diluted in OptiMEM before being combined. Transfection complexes were overlaid with $2 \times 10^5$ cells/well after 10 min. Silencing of A2M and LRP1 was confirmed using RT-qPCR and WB.

### Immunofluorescence (IF)

For microculture, 600 µL of a $5 \times 10^4$ cells/mL suspension of cells was set up on slides with Millicell EZ 8-well glass slides (Merck) and placed in an incubator at 37°C for 24 h. After incubation, cells were fixed with 4% formaldehyde for 12 min at RT and permeabilized using 0.2% Triton X-100 for 10 min. The slices were incubated at 4°C overnight with primary-specific polyclonal mouse anti-A2M (1:400 dilution; ProteinTech) and primary-specific polyclonal rabbit anti-LRP-1 (1:200; Abcam). Next, the preparations were incubated for 1 h with donkey anti-mouse secondary Alexa Fluor 568 conjugated antibodies or donkey anti-rabbit secondary Alexa Fluor 488 conjugated antibodies (1:2000 dilution; Invitrogen, Carlsbad, CA, USA) and mounted using Prolong DAPI Mounting Medium (Invitrogen, United States). Observations were made at objective 40 using a Fluoview FV3000 laser scanning confocal microscope (Olympus) coupled with Cell Sense software (Olympus).

### Statistical analysis

The Shapiro-Wilk test was used to evaluate the normality assumption of the groups examined. The Wilcoxon signed-rank test was used to compare the differences between the LSCC
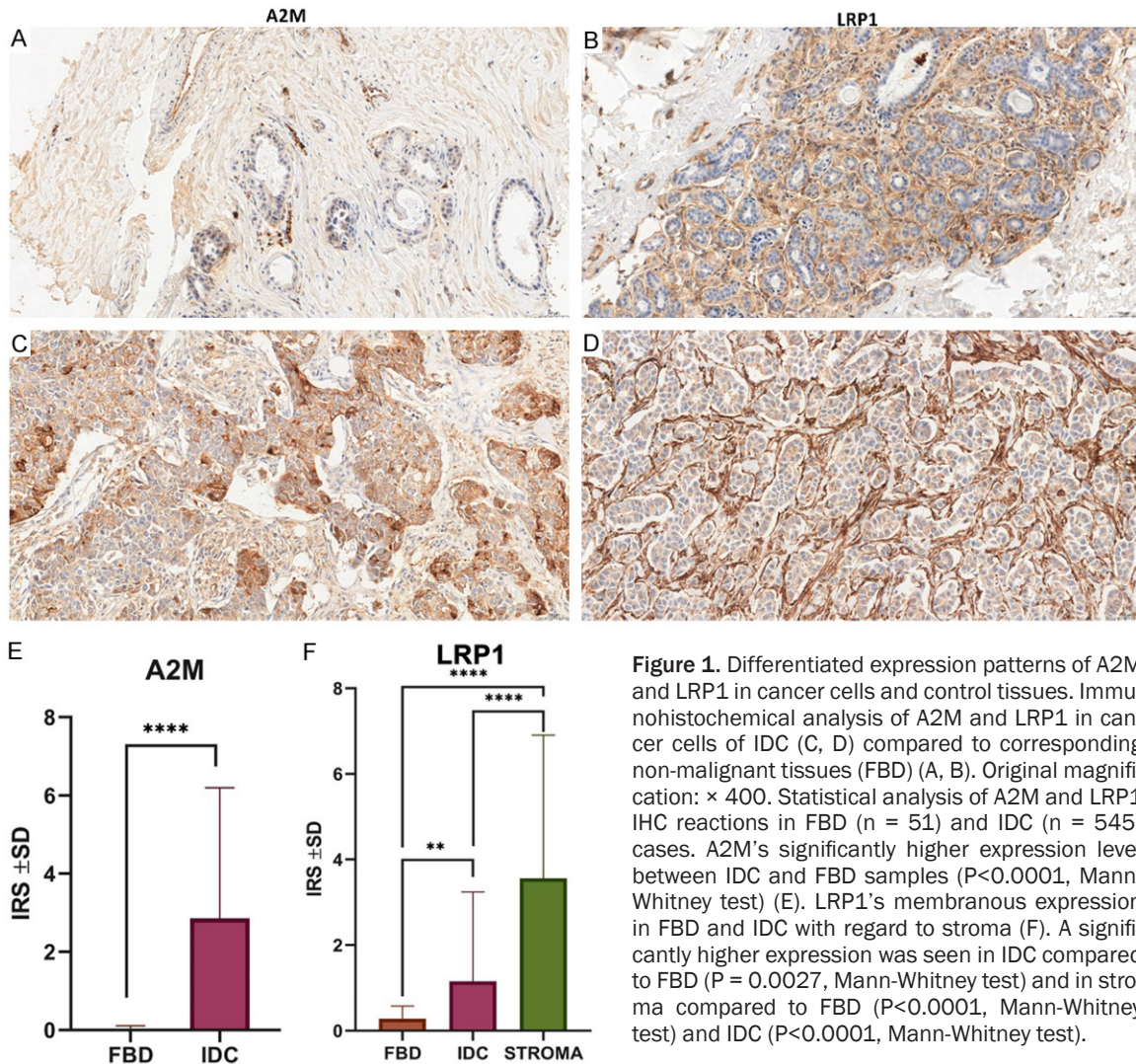
Figure 1. Differentiated expression patterns of A2M and LRP1 in cancer cells and control tissues. Immunohistochemical analysis of A2M and LRP1 in cancer cells of IDC (C, D) compared to corresponding non-malignant tissues (FBD) (A, B). Original magnification: × 400. Statistical analysis of A2M and LRP1 IHC reactions in FBD (n = 51) and IDC (n = 545) cases. A2M's significantly higher expression level between IDC and FBD samples (P<0.0001, Mann-Whitney test) (E). LRP1's membranous expression in FBD and IDC with regard to stroma (F). A significantly higher expression was seen in IDC compared to FBD (P = 0.0027, Mann-Whitney test) and in stroma compared to FBD (P<0.0001, Mann-Whitney test) and IDC (P<0.0001, Mann-Whitney test).

and NMLT groups. Additionally, Spearman correlation test was performed to analyze the existing correlations. All statistical analyses were performed using Prism 8.1.0 (GraphPad, La Jolla, CA, USA). The results were considered statistically significant at P<0.05.

## Results

### Immunohistochemistry

We observed variable expression patterns of both A2M and LRP1 and their corresponding non-cancerous tissues in breast samples (**Figure 1A-D**), lung (**Figure 3A-D**) and colorectal cancer (**Figure 5A-D**). A2M and LRP1 expression levels were observed in cancer and stromal cells. Additionally, we observed the expression of the LRP1 receptor in lung immune cells, most likely in macrophages.

### Breast cancer (IDC)

The clinicopathological features of 545 patients with invasive ductal breast carcinoma were analyzed. A2M and LRP1 expression levels were measured using immunohistochemistry. The correlation between A2M/LRP1 expression levels and the clinicopathological features and prognosis was assessed. Using IHC, we determined the cellular localization and expression levels of A2M and the membranous and stromal expression patterns of LRP1 in 51 cases of FBD and 495 cases of IDC. Cytoplasmic expression of A2M was observed in 415 (76.15%) IDC samples, compared to 5 (9.80%) in the FBD samples. Membranous expression of LRP1 was observed in 198 (36.33%) samples compared to 41 (80.40%) in the FBD samples, and stromal LRP1 expression was observed in 415 (76.15%) IDC samples.
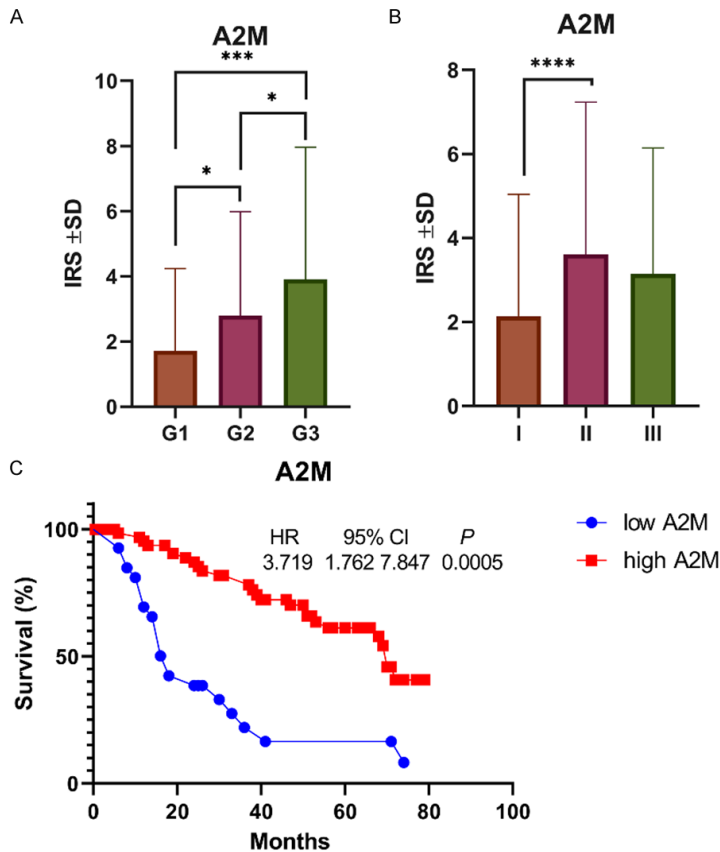
Figure 2. A2M expression in IDC with regard to malignancy grade (A) and TNM staging (B). A significantly higher expression was seen in G2 compared to G1 cases (P = 0.0132, Mann-Whitney test); in G3 compared to G1 (P = 0.0003, Mann-Whitney test) and G2 (P = 0.0146, Mann-Whitney test); and in TNM II compared to TNM I (P = 0.0029, Mann-Whitney test). Survival curve established by data obtained from KM-plot. (C) The survival time of IDC patients with high expression of A2M is higher than that of patients with low expression of A2M (P = 0.0005).

A statistical analysis showed that A2M cytoplasmic expression level was significantly higher in the IDC samples than in the FBD samples (IRS 2.7666 ± 3.2376 vs. 0.02 ± 0.2820, respectively, P<0.0001, Mann-Whitney test) (Figure 1E), and LRP1 expression levels were also significantly higher in both IDC and stromal tissues compared to the FBD samples (IRS 1.1539 ± 2.6456 vs. 3.76686 ± 3.7812 vs. 0.2802 ± 4.4387, respectively, P<0.0001, Mann-Whitney test) (Figure 1F). Moreover, statistically higher expression levels of A2M were observed when comparing the histological grades of IDC (G1 vs. G2, P = 0.0132; G1 vs. G3, P = 0.0003; G2 vs. G3, P = 0.0146; Mann-Whitney test) (Figure 2A). Statistically significant differences in A2M expression levels were observed between TNM stages I and II (P =

0.0029, Mann-Whitney test) (Figure 2B).

The Spearman correlation test revealed a high positive correlation between A2M and LRP1 membranous expression (r = 0.3425, P<0.0001), A2M and LRP1 stromal expression (r = 0.2131, P< 0.0001), and LRP1 membranous and stromal expression (r = 0.2187, P<0.0001). Additionally, a statistical analysis showed a negative correlation between ER and PR status and A2M cytoplasmic expression (A2M vs. ER: r = -0.1541, P<0.0001; A2M vs. PR: r = -0.1686, P<0.0001).

We used Kaplan-Meier to analyze the effect of A2M and LRP1 on the overall survival time of patients with IDC. We observed that higher cytoplasmic A2M expression had a negative impact on the overall survival time compared to the group with lower A2M cytoplasmic expression (P = 0.0005, HR = 0.22, 95% CI = 0.096-0.522) (Figure 2C).

*Lung cancer (NSCLC)*

The clinicopathological characteristics of 255 patients with NSCLC were examined. In 45 cases of NMLT and 255 cases of NSCLC, we used IHC to identify the cellular localization and expression levels of A2M and the membranous and stromal expression patterns of LRP1. Compared with 45 (100%) NMLT samples, 251 (98.43%) NSCLC samples showed cytoplasmic A2M expression. LRP1 expression was detected in the membranes of 242 (94.90%) NSCLC samples, as opposed to 41 (80.40%) NMLT, and in the stroma of 194 (76.08%) NSCLC samples.

A statistical analysis showed that the A2M cytoplasmic expression level was significantly higher in the NMLT samples than in NSCLC (IRS 6.3314 ± 2.2191 vs. 2.81 ± 2.1950, respectively, P<0.0001, Mann-Whitney test) (Figure 3E), but the LRP1 expression level was significantly higher in the NSCLC cases and the stroma than in NMLT (IRS 3.0942 ± 3.0423
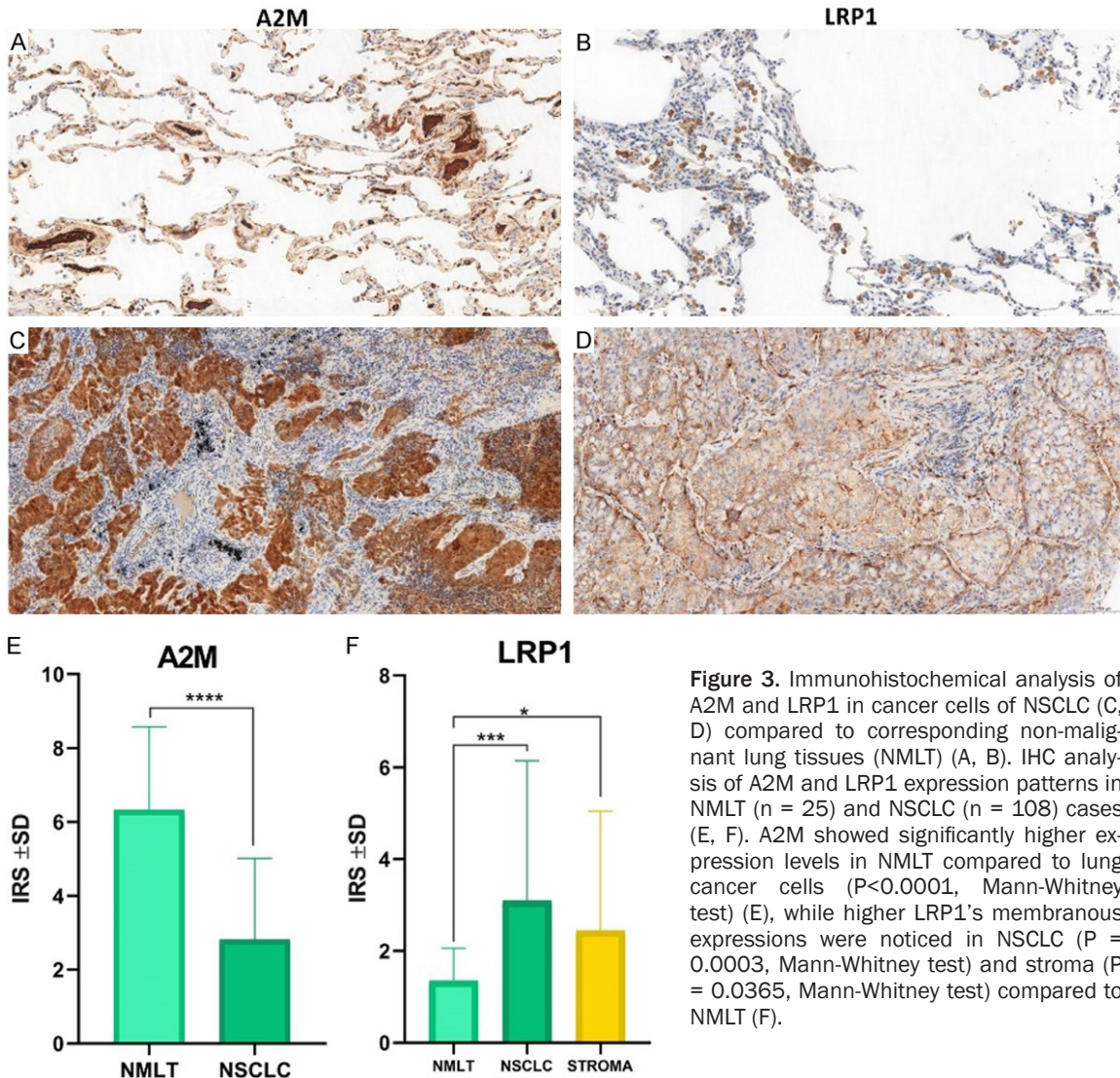
**Figure 3.** Immunohistochemical analysis of A2M and LRP1 in cancer cells of NSCLC (C, D) compared to corresponding non-malignant lung tissues (NMLT) (A, B). IHC analysis of A2M and LRP1 expression patterns in NMLT (n = 25) and NSCLC (n = 108) cases (E, F). A2M showed significantly higher expression levels in NMLT compared to lung cancer cells (P<0.0001, Mann-Whitney test) (E), while higher LRP1's membranous expressions were noticed in NSCLC (P = 0.0003, Mann-Whitney test) and stroma (P = 0.0365, Mann-Whitney test) compared to NMLT (F).

vs. 2.4418 ± 2.5968 vs. 1.3342 ± 0.7164, respectively, P<0.0001, Mann-Whitney test) (**Figure 3F**).

The Spearman correlation test revealed a high positive correlation between A2M and LRP1 membranous expression (r = 0.4383, P< 0.0001), A2M and LRP1 stromal expression (r = 0.3135, P<0.0001), and LRP1 membranous and stromal expression (r = 0.4192, P<0.0001) (**Figure 4**).

We found no evidence that A2M or LRP1 had any impact on the overall survival time of patients with NSCLC.

*Colon cancer (CC)*

In 25 cases of NMCT and 108 cases of CC, we used IHC to identify the cellular localization and expression levels of A2M and the membranous and stromal expression patterns of LRP1. A2M was detected in the cytoplasm of 106 (98.15%) CC samples as opposed to 25 (100%) NMCT samples. LRP1 expression was found in the membranes of 49 (45.37%) CC samples as opposed to 20 (80.00%) NMCT samples, as well as in the stroma of 91 (84.26%) CC samples.

A statistical analysis showed that the A2M cytoplasmic expression level was significantly higher in the CC samples than in NMCT (IRS 4.4897 ± 1.8874 vs. 2.4222 ± 0.2177, respectively, P<0.0001, Mann-Whitney test) (**Figure 5E**), and LRP1 expression levels were also significantly higher in the CC and stroma samples than in the NMCT samples (IRS 1.4022 ± 1.7608 vs. 2.9413 ± 2.2291 vs. 0.2177 ±
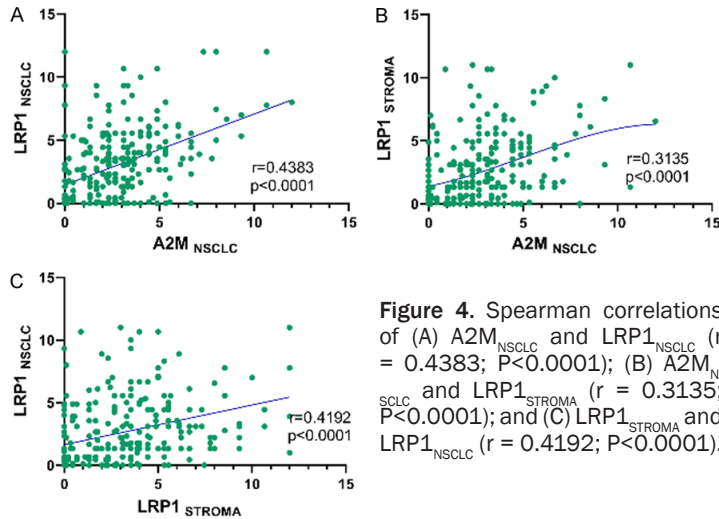
Figure 4. Spearman correlations of (A) $A2M_{NSCLC}$ and $LRP1_{NSCLC}$ (r = 0.4383; P<0.0001); (B) $A2M_{NSCLC}$ and $LRP1_{STROMA}$ (r = 0.3135; P<0.0001); and (C) $LRP1_{STROMA}$ and $LRP1_{NSCLC}$ (r = 0.4192; P<0.0001).

MB-231 (P<0.0001) cell lines (**Figure 7A** and **7B**). To confirm our RT-qPCR results, we performed a WB analysis of A2M and LRP1 protein expression levels (**Figure 7E**). **Figure 7C** shows that the expression levels of A2M were significantly lower in the Me16C cell line compared to the MCF-7 and MDA-MB-231/BO2 cell lines (P< 0.0001). Additionally, **Figure 7D** shows that the expression levels of LRP1 were statistically higher in the Me16C cell line compared to the MCF-7 (P<0.01), SK-BR-3 (P< 0.001), T-47D (P<0.01), and MDA-MB-231/BO2 (P<0.001) cell lines.

0.1895, respectively, P<0.0001, Mann-Whitney test) (**Figure 5F**).

The Spearman correlation test revealed a high positive correlation between A2M stromal levels and A2M cancer levels (r = 0.3208, P< 0.0001) (**Figure 6A**); A2M and LRP1 cancer cell expression (r = 0.3387, P<0.0001) (**Figure 6B**); and LRP1 cellular and stromal expression (r = 0.3106, P<0.0001) (**Figure 6C**).

A Kaplan-Meier curve analysis of A2M and LRP1 effects on the overall survival time of IDC patients showed a higher A2M cytoplasmic expression influence on a negative impact on the overall survival time compared to the group with a lower A2M cytoplasmic expression (P = 0.0005, HR = 3.719, 95% CI = 1.762 to 7.847) (**Figure 6D**).

*A2M/LRP1 mRNA and protein expression levels in BC, NSCLC, and CC cell lines-RT-qPCR and WB*

A real-time PCR gene expression analysis revealed that all studied breast cancer cell lines, as well as the normal breast cell line ME16C, showed significant A2M and LRP1 gene expression levels. Significantly higher expression levels of A2M were found in the MCF-7 (P<0.0001) and MDA-MB-231/BO2 (P<0.1) cell lines. On the other hand, significantly higher expression levels of LRP1 were observed in the SK-BR-3 (P<0.001), T-47D (P<0.1), and MDA-MB-231/BO2 (P<0.0001) cell lines, whereas lower expression was observed in the BT-474 (P<0.001) and MDA-

In contrast to breast cancer cell lines, lung cancer cells showed a statistically significant decrease in A2M and LRP1 mRNA levels in all studied cell lines. Compared to the reference IMR-90 cell line, A2M and LRP1 reductions in the NCI-H1703 (P<0.0001, P<0.0001), NCI-H522 (P<0.0001, P<0.001), and A549 (P< 0.0001, P<0.001) cell lines were observed (**Figure 7F** and **7G**). A WB analysis confirmed gene expression, showing that a statistical decrease in A2M protein could be seen in the NCI-H1703 (P<0.0001), NCI-H522 (P<0.0001), and A549 (P<0.0001) cell lines when compared to the lung fibroblast cell line IMR-90 (**Figure 7H**, **7J**). Furthermore, compared to IMR-90 (**Figure 7I**), LRP1 was also shown to be decreased in NCI-H1703 (P<0.001) and A549 (P<0.001) cells.

Additionally, there was significant variation in the expression levels of both A2M and LRP1 mRNAs among all studied CC cell lines. Compared to the LS180 cell line, the least aggressive of all the CC cell lines used in the experiments, both Caco-2 (P<0.001) and HT-29 (P<0.1) cells showed statistically significant increases in A2M expression. LRP1 levels were significantly decreased in the Caco-2 (P<0.1) and LoVo (P<0.001) cell lines compared to LS180 cells (**Figure 7K** and **7L**). As shown in **Figure 7M** and **7O**, Western blot showed that the Caco-2 (P<0.0001), HT-29 (P<0.001), and LoVo (P<0.001) cell lines had statistically higher levels of A2M than LS180 cells. In addition, **Figure 7N** indicates that LRP1 significantly increased protein levels in HT-29 (P<0.01) and decreased them in Caco-2 (P<0.0001) and LoVo (P<0.1) in comparison to LS180 cells.
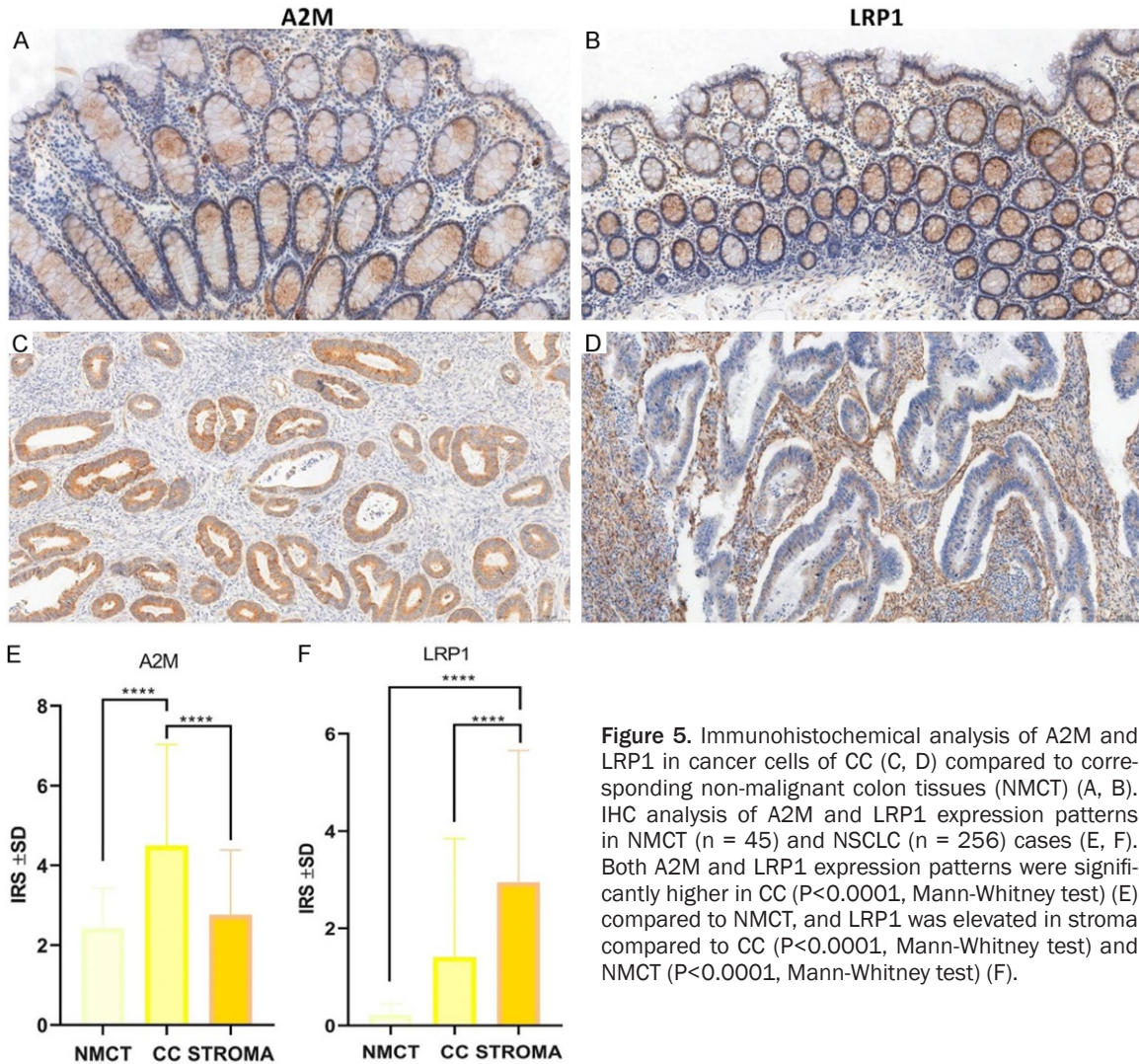
Figure 5. Immunohistochemical analysis of A2M and LRP1 in cancer cells of CC (C, D) compared to corresponding non-malignant colon tissues (NMCT) (A, B). IHC analysis of A2M and LRP1 expression patterns in NMCT (n = 45) and NSCLC (n = 256) cases (E, F). Both A2M and LRP1 expression patterns were significantly higher in CC (P<0.0001, Mann-Whitney test) (E) compared to NMCT, and LRP1 was elevated in stroma compared to CC (P<0.0001, Mann-Whitney test) and NMCT (P<0.0001, Mann-Whitney test) (F).

*siRNA silencing of A2M and LRP1 genes in breast and lung cancer cell lines*

To evaluate the effect of siRNA-mediated silencing of A2M and LRP1 mRNAs, specific siRNAs were transfected into the MCF-7 breast cancer and A549 lung cancer cell lines for 24 and 48 h. The analysis includes both negative and positive controls, as illustrated in **Figure 8K**. Transfection efficiency was measured by RT-qPCR and WB. As shown in **Figure 8**, we observed statistically significant downregulation of all silenced genes over the 24- and 48-hour periods.

The downregulation of LRP1 in the MCF-7 and A549 (**Figure 7**) cell lines resulted in a statistically significant increase in A2M levels (in both gene and protein) in the MCF-7 cell line after 48 h (P<0.0001) and in the A549 cell line after 24 h (P<0.0001) and 48 h (P<0.0001) (**Figure 8B**, **8D** and **8E**). It is interesting to note that both siRNA molecules (**Figure 8A** and **8C**) increased A2M mRNA and protein levels (P<0.0001) in the MCF-7 cell line after 48 hours of LRP1 silencing. A similar pattern was noticed in the A549 cell line after 24 h (P<0.0001) and 48 h (P<0.0001) of LRP1 silencing (**Figure 8F, 8J**). This may have happened because cancer cells responded to adverse downregulation of the LRP1 transcript. The knockdown of A2M revealed that A549 lung cancer cells responded by increasing LRP1 mRNA levels after 24 h (P<0.0001) and protein levels after 24 h (P<0.0001) and 48 h (P<0.0001) (**Figure 8G** and **8I**).
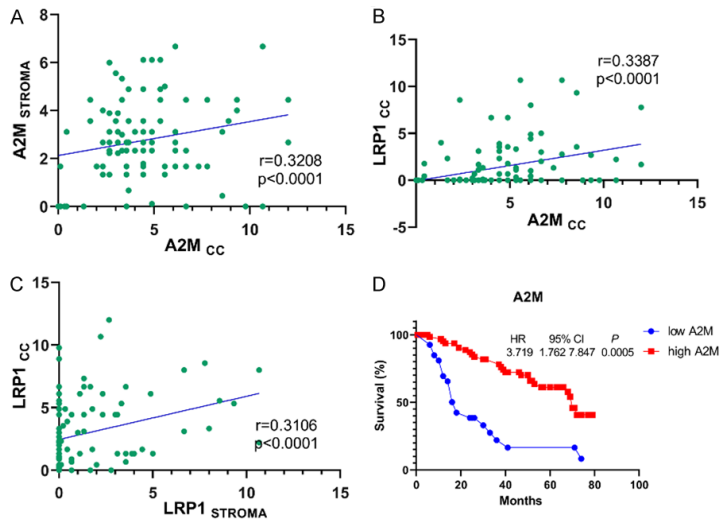
**Figure 6.** Spearman correlations of (A) A2M$_{CC}$ and A2M$_{STROMA}$ (r = 0.3208; P<0.0001); (B) A2M$_{CC}$ and LRP1$_{CC}$ (r = 0.3387; P<0.0001); and (C) LRP-1$_{STROMA}$ and LRP1$_{CC}$ (r = 0.3106; P<0.0001). (D) The survival time of CC patients with high expression of A2M is higher than that of patients with low expression of A2M (P = 0.0005).

Similar to RT-qPCR and WB expression analysis, siRNA mixtures for A2M and LRP1 were mixed with a transfection agent, incubated with the MCF-7 and A549 cell lines for 48 h, and stained for confocal microscopy analysis to evaluate the transfection efficiency. The results showed that the red fluorescence signal representing the A2M protein decreased after successful inhibition, both in the MCF-7 and A549 cell lines, and the level of LRP1 increased owing to the negative regulation of A2M. There was no evidence of LRP1 downregulation in the studied cell lines after 48 hours (**Figures 9**, **10**). Despite this, the results were in accordance with those obtained from RT-qPCR, indicating an increase in LRP1 expression levels.

## Discussion

A2M, a 750 kDa molecule that acts as a strong protease inhibitor, is detected in serum and is mostly produced by the liver. This inhibitor can enter cells and bind to numerous cytokines, such as transforming tumor necrosis factor-alpha (TNF-α), growth factor-beta 1 (TGF-β1), TGF-β2, platelet-derived growth factor-BB, nerve growth factor-beta, and basic fibroblast growth factor, and act on all classes of endoproteases. Because of its capabilities, the level of A2M in human blood declines with age, which is increasingly linked to aging processes that increase the risk of cancer. By attaching to its receptor, low-density lipoprotein receptor-related protein 1 (LRP1, also known as CD91), the fast clearance of tethered peptides and proteins is mediated. G protein-activated pathways are initiated when A2M binds to the A2M receptor, LRP. For instance, the activation of A2M by methylamine induces macrophages to increase cellular and nuclear COX-2 (Cyclooxygenase-2) when it binds to LRP receptors [85]. A2M undergoes a significant conformational change upon binding proteinases, which causes the expression of previously unnoticed receptor-binding sites on its surface. Consequently, the so-called "transformed A2M" (A2M*) binds to the LRP1 receptor. Ligation of LRP1 causes A2M-proteinase complexes to be rapidly cleared from the blood and tissues through receptor-mediated mechanisms. Nevertheless, A2M's precise role in the proliferation and metabolism of cancer cells has not yet been fully elucidated. It has been demonstrated that A2M levels in the blood and liver of long-living, cancer-resistant naked mole-rats are noticeably greater than those of humans and mice [86]. This may significantly contribute to the increased longevity and resistance to cancer in these animals and may also explain how progressive A2M deficiency may promote tumor growth, especially since most tumors grow slowly over 15 years [2-5]. We firmly believe that A2M plays an underestimated but crucial role in cancer biology because of its capacity to function as a signaling molecule, growth factor transporter, and modulator of protease activity.

It has been demonstrated that reducing A2M levels through exogenous protease injection can reduce TGF-β levels and alter the kinetics of tumor growth [87]. According to these findings, elevated blood levels of the PSA-A2M complex in patients with prostate cancer may change the concentrations of vital growth and survival variables that could influence prostate cancer progression. Changes in these protein levels may also affect certain signs and symptoms of advanced or terminal prostate cancer,
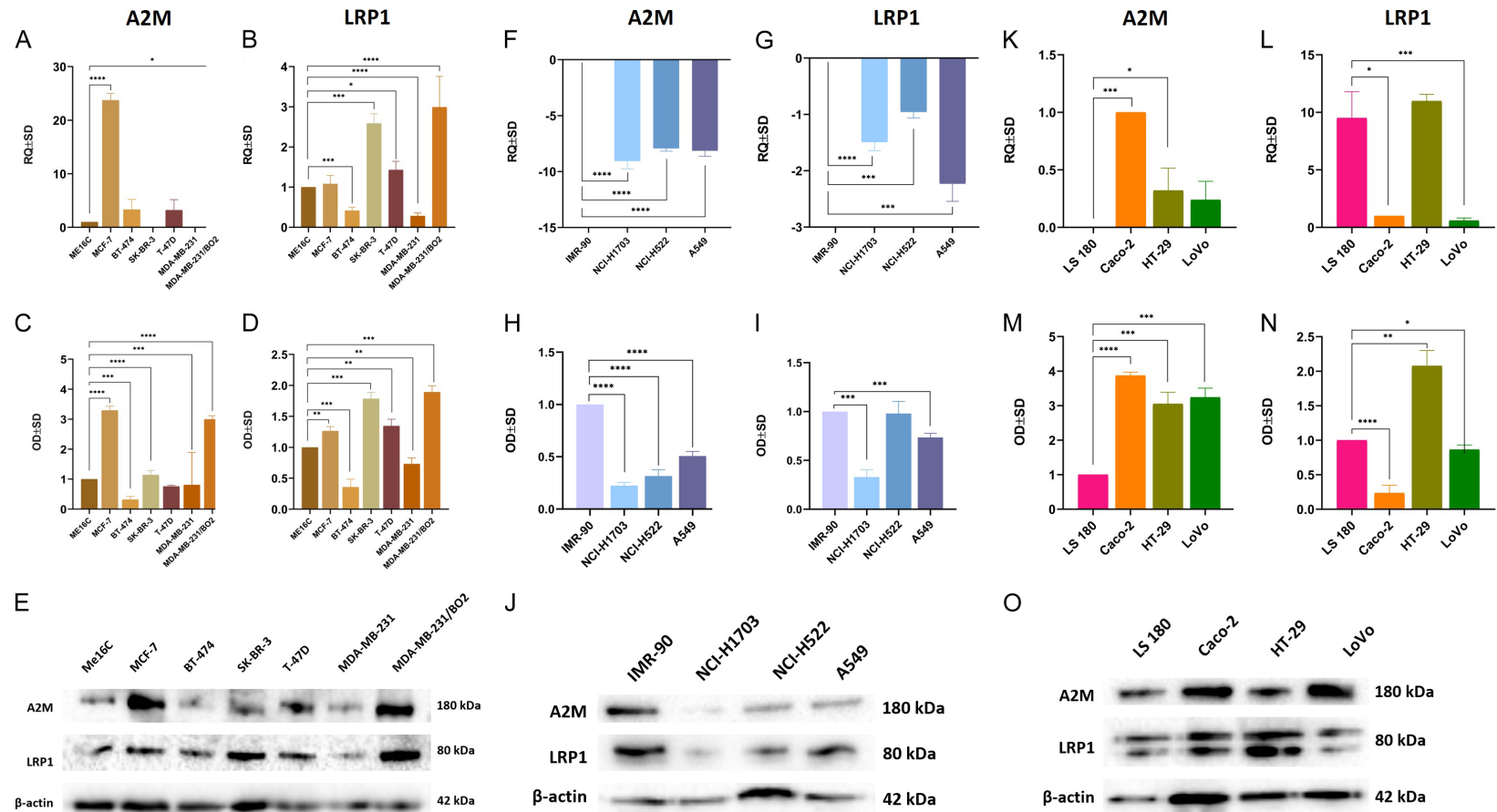
**Figure 7.** Relative expression levels of A2M and LRP1 in breast cancer cell lines (A, B), lung cancer cell lines (F, G), and colorectal cancer cell lines (K, L). Data are the mean ± SD of triplicate determinants. Western blot analysis of A2M and LRP1 in breast cancer cell lines (C, D), lung cancer cell lines (H, I), and colorectal cancer cell lines (M, N). As an internal control, a β-actin protein was used (E, J, O). Data show the average standard deviation of three independent experiments. *P<0.1; **P<0.01; ***P<0.001; ****P<0.0001.
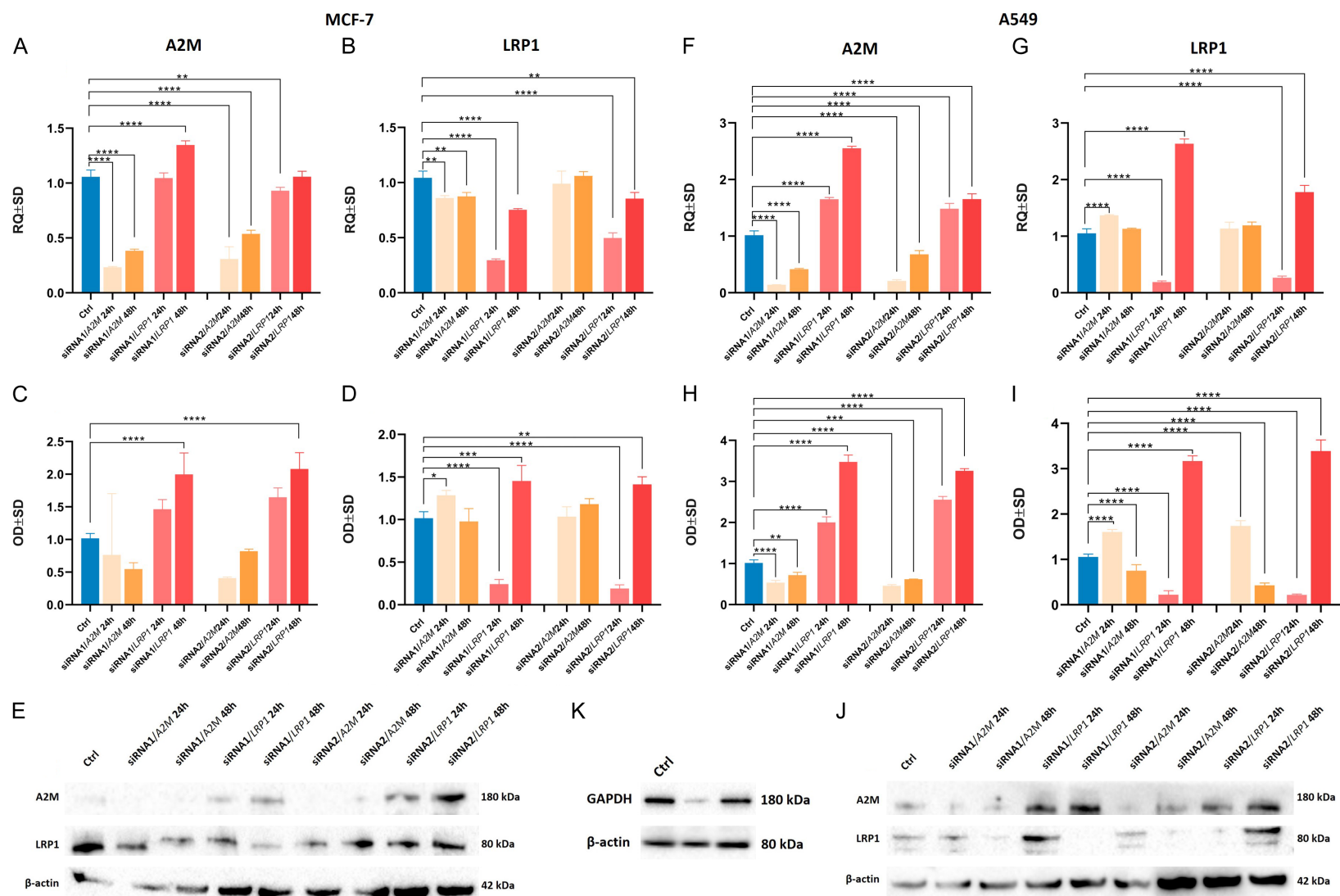
**Figure 8.** Relative levels of A2M and LRP1 mRNAs in MCF-7 IDC cell line and A549 NSCLC cell line transfected with siRNAs against A2M and LRP1. Cells were transfected with the siRNAs for A2M and LRP1 and incubated for 24 h and 48 h. Relative expression levels (RQs) of A2M in MCF-7 (A) and A549 (F) cells, and LRP1 mRNA after silencing in MCF-7 (B) and A549 (G) cells. (C and H) show protein expression analysis of A2M, whereas (D and I) show the analysis of LRP1 in MCF-7 and A549 cell lines. Western blot analysis measured the effects of siRNA-mediated knockdown of A2M and LRP1 in MCF-7 (E) and A549 (J) cell lines. GAPDH was used as an internal control (K). Data represent the mean and standard deviation of three independent experiments. Comparisons between groups were done using Student's t-test: *$P<0.1$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.
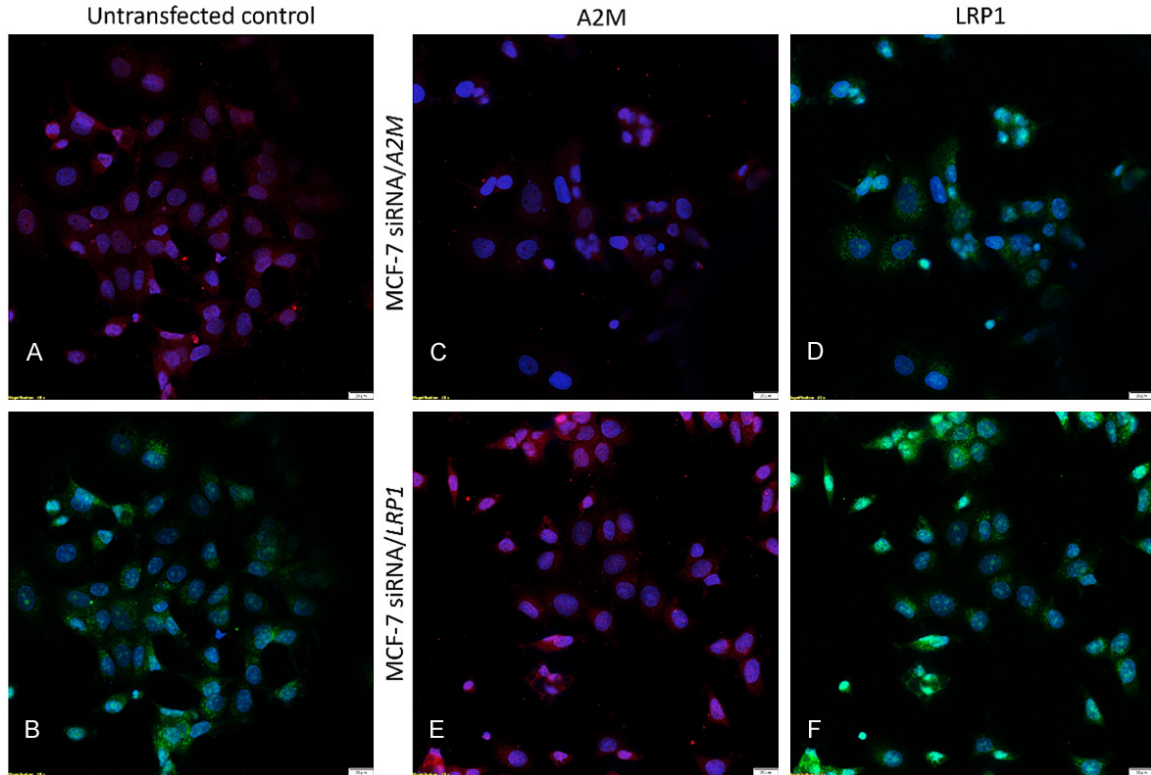
**Figure 9.** Inhibition of gene expression using siRNA transfection. Immunofluorescence staining of A2M and LRP1 proteins in human breast cancer cell line MCF-7 post-transfection. Protein reduction was sustained 48 h post-siRNA transfection. A2M was immunostained red, LRP1 was immunostained green, and nuclei were stained with DAPI (blue). Untransfected control labeled with A2M (A) and LRP1 (B) antibodies. MCF-7 siRNA/A2M cell line stained with A2M (C) and LRP1 (D). MCF-7 siRNA/LRP1 cell line stained with A2M (E) and LRP1 (F).

including increased discomfort, cachexia, and exhaustion [54]. Reduced levels of total A2M and higher concentrations of PSA bound to A2M have the potential to interfere with blood protease clearance and inactivation, which may result in abnormalities in the complement and clotting-system protease cascades.

Our research on three different types of human cancers revealed that the expression of A2M and its binding receptor, LRP1, varies among cancer cells. Breast cancer cells show a decreasing trend in A2M expression, which is correlated with cancer cell aggressiveness. Similarly, the expression levels of A2M are much lower in lung cancer cells than in lung fibroblasts. Unfortunately, we did not examine serum levels of A2M, which would have greatly enhanced the findings of this study and brought us closer to understanding the role of A2M in the development of cancer. LRP1 levels are considerably lower in tumorous lung tissue than in non-malignant lung tissue. In a study

group of 256 patients, lower LRP1 expression in lung adenocarcinoma was associated with a worse clinical prognosis.

It is noteworthy that A2M expression levels are higher in the tissues of CC patients, similar to those of IDC patients, compared to the corresponding non-malignant tissues. This finding contrasts with an analysis that linked a higher expression of A2M in cancerous tissue with a longer survival time for patients with IDC and CC. These findings demonstrate the extremely nuanced functions of A2M and its receptor, LRP1, in the development of cancer. An analysis of the relationships between A2M and LRP1 in breast, lung, and colorectal malignancies clearly showed that A2M should be regarded as a pan-endoprotease inhibitor implicated in a broad range of biological and physiological processes.

Moreover, the results of the siRNA knockdown experiments revealed that successful inhibition
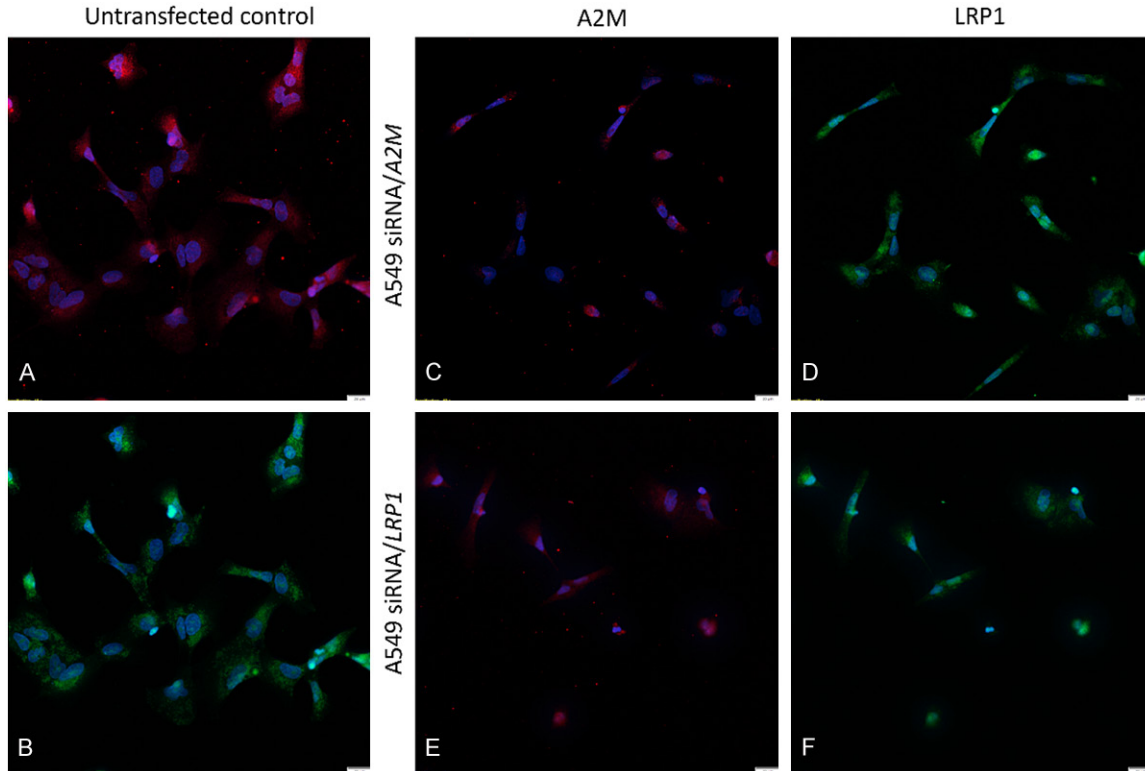
**Figure 10.** Inhibition of gene expression using siRNA transfection. Immunofluorescence staining of A2M and LRP1 proteins in human lung cancer cell line A549 post-transfection. Protein reduction was sustained 48 h post-siRNA transfection. A2M was immunostained red, LRP1 was immunostained green, and nuclei were stained with DAPI (blue). Untransfected control labeled with A2M (A) and LRP1 (B) antibodies. A549 siRNA/A2M cell line stained with A2M (C) and LRP1 (D). A549 siRNA/LRP1 cell line stained with A2M (E) and LRP1 (F).

of *LRP1* elevated *A2M* mRNA and protein levels. This may indicate that direct interactions between A2M and LRP1 receptors may be crucial in cancer physiology. Although the exact mechanism by which this finding was made is unknown, we hypothesized that it may have been caused by changes in the LRP1/A2M complex's binding capacities and, therefore, changes in the endocytosis process. Nevertheless, further investigations are required to fully understand this mechanism.

A2M has a significant impact on aging and cancer development. A2M blood levels in humans are adversely associated with aging, falling from 4 mg/mL at birth to 1.5 mg/mL in the elderly [88]. Thus, its role in maintaining blood homeostasis and preventing diseases associated with aging is clinically important. In conclusion, our findings provide further evidence that A2M and LRP1 play a role in cancer development. It is crucial to investigate A2M's role and potential binding partners and receptors

that may be involved in carcinogenesis because, in human blood and tissues, it can bind and inhibit the activities of most proteinases, as well as numerous growth factors, hormones, and cytokines.

## Conclusions

Our findings support the relevance of A2M and its receptor, LRP1 (CD91), in cancer development and tumor-stroma communication, which leads to the control of cancer growth, cancer cell death, and the anti-inflammatory cytokine signaling response. Our findings emphasize the diagnostic utility of A2M and LRP1 in patients with BC, NSCLC, and CC. Nonetheless, further research is needed to determine the precise mechanisms of the direct interaction between A2M and LRP1 that underlies the pathophysiology of human malignancies. Restoring A2M homeostasis in the tumor to that found in healthy tissues may impair the ability of the tumor to evade immune control. Confirmation

of A2M's significance in cancer treatment may contribute to the development of novel anti-cancer treatments based on A2M.

## Disclosure of conflict of interest

None.

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