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LETTERS TO THE EDITOR

Dll1/Notch activation accelerates multiple myeloma disease development by promoting CD138 + MM-cell proliferation

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Multiple myeloma (MM) is a B-cell malignancy characterized by the accumulation of aberrant plasma cells in the patients' bone marrow (BM). Relapse often occurs after recovery of MM patients from chemotherapy and has become one of the greatest challenges in MM treatment. The BM microenvironment has a critical role in MM-cell growth and survival. Several pathways, such as MAPK, p53, JAK/STAT3, RANK/RANKL/OPG, NF-κB and WNT are involved in the process of MM development. The Notch signaling is a highly conserved pathway that regulates cell-fate determination, stem cell self-renewal, proliferation and apoptosis.[1] In this pathway, four receptors (Notch1-4) and several ligands (Jag1-2, Dll1, Dll3-4) have been described. These receptors and ligands were detected both in MM cells and in BM stromal cells.[2–5] Furthermore, it has been described in human MM that Jag1- and Jag2-induced Notch activation resulted in MM-cell proliferation and that Notch1 signaling is involved in drug resistance.[2–4] Controversially, Zweidler-McKay *et al.*[6] claimed that constitutive activation of Notch by Notch intracellular domain (NICD1-4) transduction inhibited malignant B-cell growth and induced apoptosis. The role of the Dll1/Notch pathway in myeloma cell proliferation and clonogenic growth as well as disease engraftment has not yet been studied. We investigated this pathway using the 5T33MM murine model that mimics the human MM disease closely as described previously.[7] The human MM cell lines MMS-1 and LP-1 were used to confirm our obtained mouse data.

In this study, we first investigated whether Notch receptors were expressed on murine 5T33MMvt cells, and human MMS-1 and LP-1 cells. Notch1 and Notch2 receptors were expressed on 5T33MMvt cells, and human MMS-1 and LP-1 MM cells as demonstrated by Fluorescence-activated cell sorting (FACS) (Figure 1a), and hereby confirmed the western blot data described by others.[3] It was previously reported that Dll1 (delta-like 1) is expressed by BM stromal cells.[2] We confirmed these data by detecting the expression of Dll1 by RT – PCR (Figure 1b) and western blot (Figure 1c) using BM stromal cells from both normal donors and MM patients. Next, we used the MS5.Dll1 stromal cell

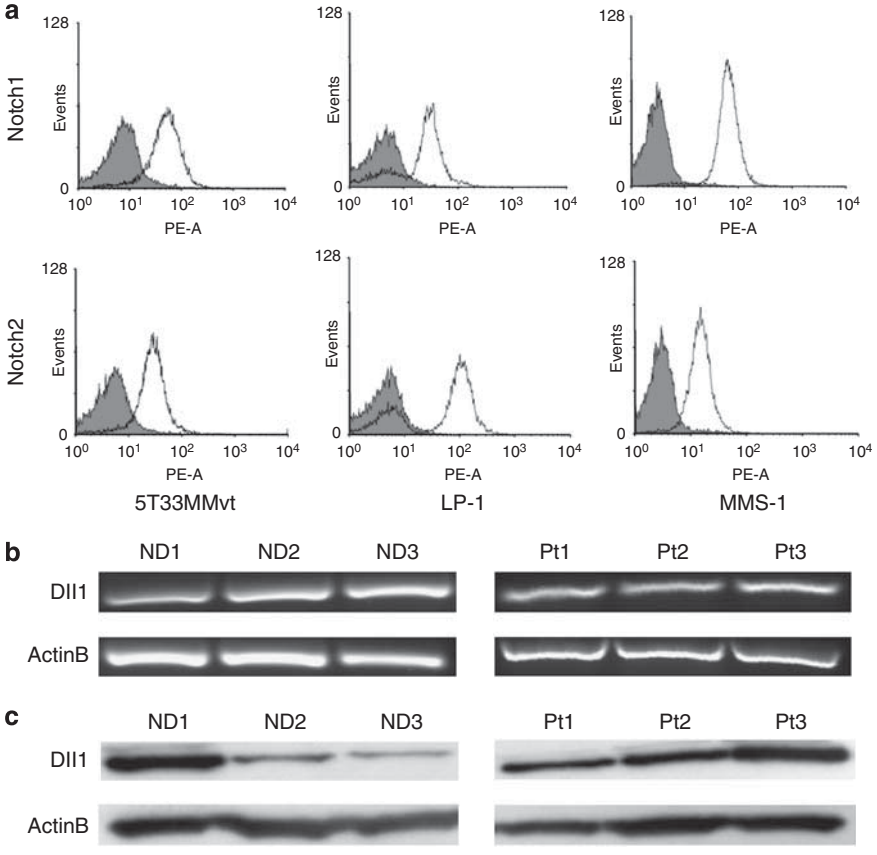

Figure 1. Expression of the Notch receptors in MM cells and Dll1 ligand in BM stromal cells. (**a**) FACS analysis of the expression of Notch1 and Notch2 receptors on murine 5T33MMvt, and human LP-1 and MMS-1 cells. Filled histograms represent the isotype control. Open graph represents specific antibodies. Representative results of three independent experiments are shown. (**b**) Expression of the Notch ligand Dll1 was investigated by RT – PCR in BM stromal cells derived from normal donors (ND) and MM patients (Pt). (**c**) Expression of the Notch ligand Dll1 was investigated by western blot in BM stromal cells derived from normal donors (ND) and MM patients (Pt).

line,[8] which is genetically modified to have a high constitutive expression of human Dll1 (a highly conserved protein from mouse to human), to activate the Notch pathway in these murine and human MM cells in cocultures. We investigated the expression of the Notch downstream genes (Hes1, Hes5, Hey1, Hey2, HeyL) in MM cells cocultured with MS5.Dll1 stromal cells to evaluate the

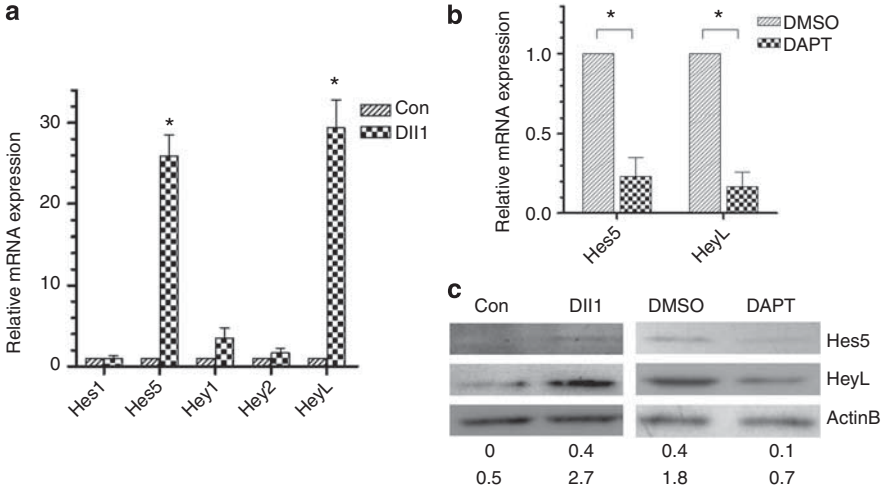

Figure 2. Activation and inhibition of the Notch pathway in 5T33MMvt cells. (**a**) Real-time PCR was performed for Notch pathway downstream genes (Hes1, Hes5, Hey1, Hey2, HeyL) after Dll1/Notch interaction. Con and Dll1 indicate cocultures of 5T33MMvt cells with MS5 or MS5.Dll1 stromal cells for 48 h, respectively. Results are shown as mean ± s.d. ($n = 3$). *Indicates $P < 0.05$ (Mann–Whitney *U*-test). (**b**) mRNA expression of Hes5 and HeyL after 10 μM DAPT treatment for 48 h. DMSO is the control. Results are shown as mean ± s.d. ($n = 3$). (**c**) Western blot for Hes5 and HeyL (Abcam, UK) expression after Dll1/Notch interaction or DAPT treatment for 48 h when cocultured with MS5.Dll1 stromal cells. The numbers below refer to the optical density of Hes5 and HeyL as measured with ImageJ programme. One experiment representative of three is shown.

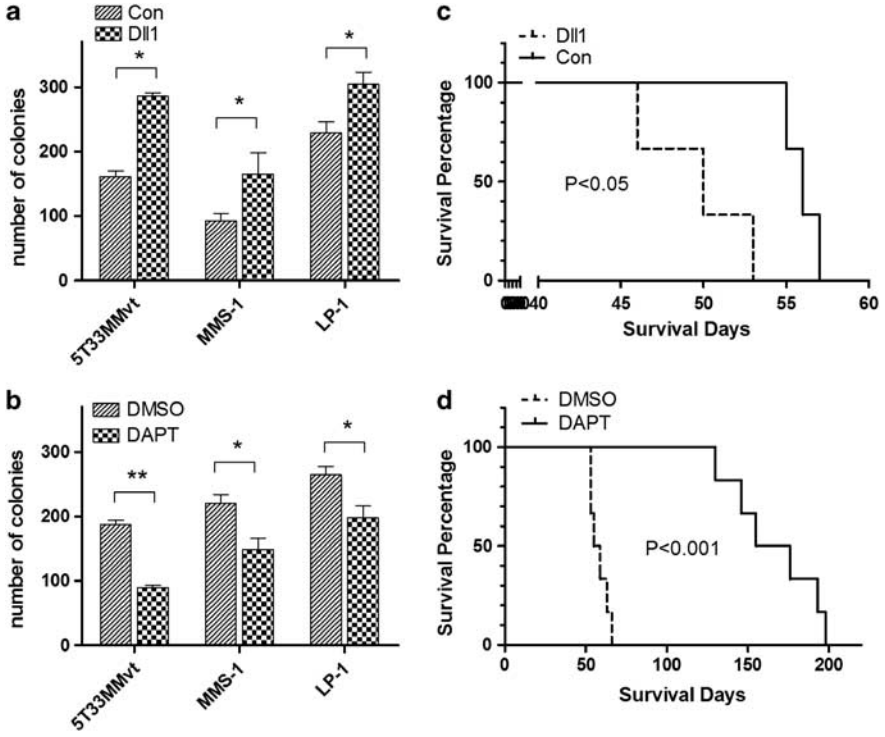

Figure 3. Dll1/Notch interaction promotes MM clonogenic growth and accelerates MM initiation whereas DAPT inhibits MM self-renewal ability and significantly delays MM development. (**a**) CFC assays were performed after cocultures of MM cells with MS5 (Con) or MS5.Dll1 (Dll1) stromal cells for 4 days. Cells were plated at a concentration of 2500 per ml (5T33MMvt), 30 000 per ml (MMS-1) and 10 000 per ml (LP-1). The number of colonies were counted and presented as mean ± s.d. ($n = 3$). (**b**) 5T33MMvt cells were treated with 10 μM DAPT for 4 days; MMS-1 and LP-1 cells were cocultured with MS5.Dll1 stromal cells and treated with 10 μM DAPT for 4 days and plated in CFC assay for assessing clonogenic ability. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively (Mann–Whitney *U*-test). (**c**, **d**) Kaplan–Meier survival analysis. The *x* axis represents the survival days from injection to the development of hind leg paralysis and the *y* axis represents the survival percentage. (**c**) 5T33MMvt cells cocultured with MS5 or MS5.Dll1 stromal cells for 6 days were injected intravenously into naive mice ($n = 3$) to investigate MM *in vivo* engraftment. (**d**) 5T33MMvt cells pretreated with DMSO or 10 μM DAPT for 6 days were injected intravenously into naive mice ($n = 6$).

activation of the Notch pathway. The PCR primer sequences are listed in Supplementary Table 1a and 1b and real-time PCR was performed using SYBR Green (Fermentas, UK). We found that Hes5 and HeyL were significantly upregulated after Dll1/Notch interaction for 48 h in 5T33MMvt cells (Figure 2a), which was also confirmed by western blot (Figure 2c). A similar upregulation of Hes5 and HeyL mRNA was observed when mouse 5T33MMvt cells were stimulated with mouse Dll1 ligand (150 ng/ml). In MMS-1 cells, Hes1, Hes5 and HeyL were upregulated whereas in LP-1 cells, all these five genes were upregulated (data not shown). It indicated that cocultures of MM cells with MS5.Dll1 stromal cells could activate the Notch pathway in MM cells. Then, we investigated whether *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT, Gamma secretase inhibitor, a Notch pathway inhibitor) (Sigma-Aldrich, Bornem, Belgium) could inhibit the Notch activation induced by the Dll1/Notch interaction. Previous published studies used a DAPT concentration ranging from 5 to 50 μM in myeloma cells.[3,7] We performed a titration assay (data not shown) and chose 10 μM DAPT for further experiments. Treatment of MM cells with 10 μM DAPT for 48 h could inhibit the expression of the Notch downstream genes both in murine 5T33MMvt cells (Figure 2b) and in human MMS-1 and LP-1 cells (data not illustrated).

Next, we investigated the involvement of the Notch pathway in MM-cell clonogenic growth *in vitro*. 5T33MMvt cells cocultured with MS5 or MS5.Dll1 stromal cells for 2, 4 and 6 days were collected and plated in MethoCult medium (StemCell Technologies, Grenoble, France) for Colony-forming cell assays (CFC assay). We detected an increased number of colonies in the MS5.Dll1-stimulated group compared with the MS5 group (Figure 3a, only 4 days data are shown). Cells from the colonies were positive for the 5T33MM idiotype (as detected by FACS) confirming that the colonies are formed by 5T33MMvt cells. In addition, MS5 or MS5.Dll1 stromal cells alone could not form any colonies. Similar results were obtained when using human MMS-1 and LP-1 MM cell lines (Figure 3a) demonstrating that Dll1/Notch interaction could promote MM-cell clonogenic growth. Conversely, the MM clonogenic ability was inhibited and fewer colonies were formed when blocking constitutive Notch signaling with 10 μM DAPT (pretreated before CFC assay) for 4 days in 5T33MMvt, MMS-1 and LP-1 MM cells (Figure 3b).

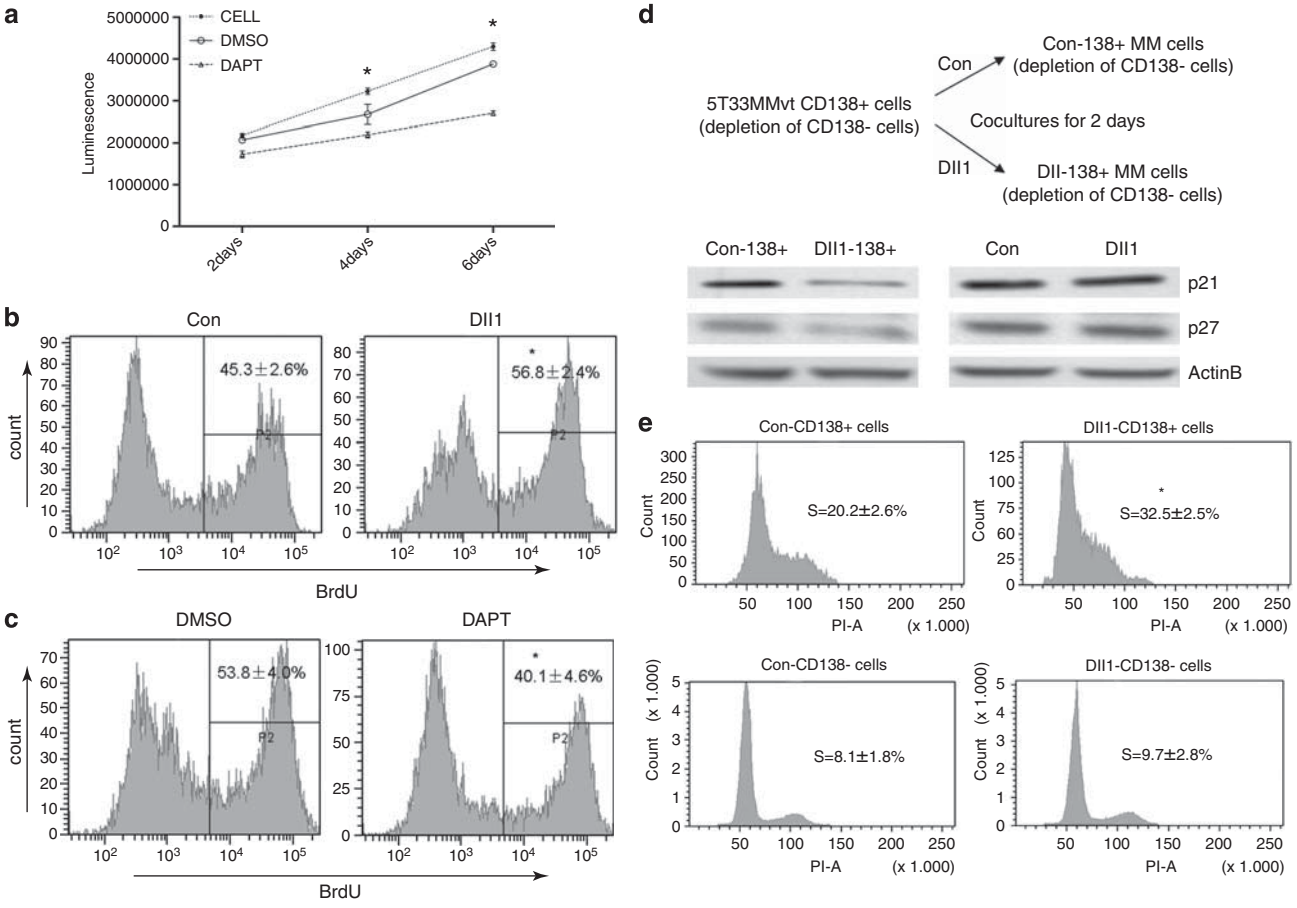

Figure 4. Role of the Dll1/Notch pathway in MM proliferation. (**a**) Cell viability assay (Cell Titer Glo, Promega) of 5T33MMvt cells treated with DMSO or DAPT for 2, 4 and 6 days ($n = 3$). (**b and c**) BrdU-uptake was analyzed by flow cytometry to investigate murine 5T33MMvt-cell proliferation after Dll1/Notch interaction and DAPT treatment. Con and Dll1 represent cocultures of MM cells with MS5 or MS5.Dll1 stromal cells for 2 days, respectively. DMSO and DAPT represent cocultures of 5T33MMvt cells with MS5.Dll1 stromal cells, and treated with DMSO and 10 μM DAPT for 2 days, respectively. One representative experiment for three is given. Percentage of BrdU-positive cells is shown as mean ± s.d. (three independent experiments). (**d**) Western blot for p21 and p27 (Cell signaling, Danvers, MA, USA) expression after Dll1/Notch interaction. Con and Dll1 represent total population of 5T33MMvt cells cocultured with MS5 or MS5.Dll1 stromal cells for 2 days, respectively. Con-138+ and Dll1-138+ represent CD138+ sub-population (stained with anti-mouse CD138-PE (BD Biosciences, Franklin Lakes, MJ, USA) followed by anti-PE microbeads (Miltenyi Biotec, Leiden, The Netherlands) and sorted by MACS) of 5T33MMvt cells cocultured with MS5 or MS5.Dll1 stromal cells for 2 days, respectively. (**e**) Cell cycle distribution of 5T33MMvt CD138+ and CD138− cells after Dll1/Notch interaction for 2 days. Percentage of cells in S phase is shown as mean ± s.d. ($n = 3$). * indicates $P < 0.05$ (Mann–Whitney *U*-test) compared with DMSO.

Subsequently, we investigated the role of Dll1/Notch interaction in MM disease development and *in vivo* engraftment in the 5T33MM murine model. 5T33MMvt cells cocultured with MS5 or MS5.Dll1 stromal cells for 6 days were harvested, counted and viability was tested with the Cell Titer-Glo assay (Promega, Leiden, The Netherlands). MM cells (200 μl) were injected intravenously into naive C57Bl/KaLwRij mice at 0.5 million cells/mouse. Mice injected with Dll1-activated 5T33MMvt cells developed disease faster than those injected with the control cells (Figure 3c), and also had a higher serum M spike concentration (1.98 ± 0.44 g/dl, $n = 3$) compared with control mice (0.95 ± 0.02 g/dl, $n = 3$), suggesting that Notch activation not only promoted MM clonogenic growth *in vitro* but also accelerated MM development *in vivo*. Furthermore, 5T33MMvt cells pretreated with 10 μM DAPT (Dimethyl sulfoxide (DMSO) as control) for 6 days, to inhibit constitutive Notch pathway activation, were injected into naive mice. DMSO- and DAPT-treated cells had the same viability when injected, as analyzed by the Cell Titer-Glo assay. After an average of 59 days, the DMSO control group became sick with an average M-compound of 1.78 ± 0.14 g/dl ($n = 6$), whereas in the DAPT group, all mice looked healthy and no serum M compound could be detected at that time. The DAPT-treated group did develop MM disease, at a significantly later time point (average of 161 days after injection) with a lower M-compound concentration (0.35 ± 0.13 g/dl, $n = 6$), suggesting that the Notch pathway has a critical role in MM initiation and development (Figure 3d).

We investigated why Dll1/Notch pathway could promote MM clonogenic growth and accelerate disease development *in vivo*. As tumor growth is the result of MM-cell proliferation and reduced apoptosis, we analyzed the effect of the Dll1/Notch pathway on these processes. Treatment of 5T33MMvt cells with 10 μM DAPT for 2, 4 and 6 days showed that MM-cell growth was indeed suppressed as measured by Cell Titer-Glo assay (Figure 4a). However, it was not caused by the induction of apoptosis by DAPT as AnnexinV and 7-aminoactinomycinD (7-AAD) staining showed only a few apoptotic cells after DAPT treatment for 2 days in 5T33MMvt, MMS-1 and LP-1 cells (data not shown). We investigated MM-cell proliferation by incubating MM cells with Bromodeoxyuridine (BrdU) for 18 h. It revealed that Dll1/Notch interaction could indeed increase MM-cell proliferation rate in 5T33MMvt cells (Figure 4b), which can be suppressed by DAPT treatment (Figure 4c). Similar results were obtained in MMS-1 and LP-1 cells (data not shown) showing that Dll1/Notch interaction could promote MM-cell proliferation. We further analyzed MM cell cycle distribution to confirm the above data. Cell cycle distribution analysis showed that the percentage of cells in the S phase increased slightly after the Dll1/Notch interaction for 2 days in 5T33MMvt (from $22.1 \pm 2.2\%$ to $25.5 \pm 2.8\%$), MMS-1 (from $21.6 \pm 2.2\%$ to $25.2 \pm 3.4\%$) and LP-1 (from $19.1 \pm 1.2\%$ to $22.9 \pm 0.4\%$) whereas DAPT could decrease the percentage compared with DMSO control (data not shown). As cell cycle inhibitory proteins (p21 and p27) are two key regulators for the S phase checkpoint, we investigated whether these molecules were involved. We found that there were no differences between total population of 5T33MMvt cells before and after Dll1/Notch interaction (Figure 4d). However, when we focused on CD138+ MM cells, which were also the main population, we found that Dll1/Notch interaction could reduce the expression of p21 and p27 (Figure 4d) in CD138+ 5T33MMvt cells accompanied by an increase in the percentage of cells in the S phase in CD138+ MM cells (Figure 4e). In contrast, CD138− MM cells were relatively quiescent and their cell cycle distribution was not affected by Dll1/Notch interaction (Figure 4e).

Taken together, our experiments demonstrate that Dll1/Notch interaction could increase MM clonogenic growth *in vitro* and accelerate disease development *in vivo*, mainly by increasing MM-cell proliferation rather than preventing MM-cell apoptosis. Furthermore, Dll1/Notch interaction promotes MM-cell proliferation predominantly in CD138+ MM cells by reducing the expression of p21 and p27, thus accelerating MM cell cycling. This is in agreement with our data that 5T33MM CD138+ cells have a greater *in vitro* clonogenic and *in vivo* engraftment capacity compared with CD138− cells (manuscript submitted). More importantly, we here report for the first time that the Notch pathway is involved in MM engraftment and development *in vivo*. Inhibiting Notch signaling as a whole could significantly delay MM initiation and development, which provides an interesting strategy to prevent or delay MM relapse by combination of chemotherapeutics with a Notch pathway inhibitor.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)