Improving the Efficacy of Vincristine for the Treatment of Childhood Acute Lymphoblastic Leukemia in a Murine Model

Objective: Pro-B Acute Lymphoblastic Leukemia (Pro-B ALL) is the most prevalent form of acute leukemia diagnosed in children and represents the most common malignancy of childhood. Despite the advances in the treatment of ALL, many children with ALL suffer from serious side effects of the treatment and even die due to relapse. Therefore, it is necessary to develop more effective but less toxic drugs for the treatment of ALL.

Methods: In the present study we evaluated the antitumor effects of Phosphoethanolamine (Phos), alone and in combination with the chemotherapeutic agent Vincristine (VCR) in an in vivo murine model injected with Pro-B Acute Lymphoblastic Leukemia (Pro-B ALL) and then investigate its effect on the efficacy of chemotherapeutic agent Vincristine (VCR) in combination therapy.

Results: Herein, we showed that although phos is able to increase the percentage of apoptotic leukemic clones sequestered by the spleen, but the ability of phos alone to reduce the percentage of leukemic clones in vivo in peripheral blood of RS4;11 engrafted mice was not observed. Phos showed a synergistic effect on VCR treatment and increased the percentage reduction of leukemic cells compared to treatment with VCR alone. Also, this combined treatment showed VCR 0.1 mg.Kg⁻¹ is able to be as effective as VRC 0.5 mg.Kg⁻¹ treatment in terms of percentage of leukemic cells in peripheral blood.

Conclusion: Phos showed that it can improve the efficiency of VCR and thus reduce the necessary dose for antitumor effect. This suggests its use as an adjuvant agent that may reduce the toxicity associated with cancer treatment.

Keywords: Acute Lymphoblastic Leukemia, Phosphoethanolamine, In Vivo, Murine Model, Vincristine

Abstract

Acute lymphoblastic leukemia (ALL) is a hematologic disease characterized by the malignant transformation and proliferation of immature lymphoid cells in the bone marrow, peripheral blood, and sometimes other organs. The disease is broadly classified into two lineages, T-lineage ALL (T-ALL) and B-lineage ALL. ALL is the most common pediatric malignancy, which representing about 25% of all cancers in childhood. The incidence of ALL is higher in children aged 2-5 years, with the peak incidence occurring at age 2 years.

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In the present study, we evaluated the antitumor effects of Phosphoethanolamine (Phos), alone and in combination with the chemotherapeutic agent Vincristine (VCR) in an in vivo murine model injected with Pro-B Acute Lymphoblastic Leukemia (Pro-B ALL) and then investigate its effect on the efficacy of chemotherapeutic agent Vincristine (VCR) in combination therapy.

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idence of childhood ALL is 3–4 cases per 100,000 patients aged 0-15 years per year with a peak incidence occurring at 2 to 5 years of age, while for patients older than 15 years, it is about 1/100,000 cases. Apart from age, the incidence of ALL also depends on race and sex. Childhood ALL occurs more than twice as often in whites as in blacks. Also, boys are more affected than girls (55% to 45%). Different genetic and environmental factors are related to increased risk of ALL. Genetic syndromes like Down syndrome, neurofibromatosis type I, Fanconi anemia, ataxia-telangiectasia, Bloom syndrome and Niemegen breakdown syndrome and also environmental risk factors like exposure to ionizing radiation and some chemicals such as solvents and pesticides, are associated with an increased risk of ALL. Over the past decades, ALL treatment has improved due to the adapting therapy to relapse risk, improved supportive care, and optimized chemotherapy drugs. Currently, the five-year event-free survival rate is about 90% for children with ALL and about 50% for adults. ALL treatment approach is one of the most intensive and complex cancer therapy programs. Although treatment regimens and drug selection, dosage schedules, and treatment periods vary between adult and children patients, and different ALL subtypes, the basic principles of treatment are the same. The most commonly used treatments for ALL patients are mainly based on a 4/5 drug induction regimen that includes a combination of vincristine, a corticosteroid (e.g., dexamethasone or prednisone), an anthracycline (e.g., doxorubicin or daunorubicin), and L-asparaginase, with or without cyclophosphamide.

Vincristine sulfate (VCR), also known as leurocristine, is a purified alkaloid obtained from the Madagascar periwinkle plant, Catharanthus roseus. Vincristine by binding to the β-subunit of tubulin, lead to microtubule depolymerization. This disruption in the meiotic spindle causes mitosis inhibition and cell death. VCR that was discovered in 1960, is currently one of the most important chemotherapeutic drugs that is used in the treatment of various diseases like non-Hodgkin and Hodgkin lymphomas, acute lymphoblastic leukemia (ALL), Ewing’s sarcoma, brain tumors, wilms tumor, neuroblastoma and rhabdomyosarcoma. However, like many other anticancer drugs, the therapeutic potential of VCR is limited by its dose-dependent side effects including myelosuppression, neurotoxicity and gastrointestinal symptoms. These side effects can have a serious impact on life quality and can lead to delays in treatment and even to discontinuation of Vincristine therapy.

The primary amines phosphoethanolamine (Phos) is the main component involved in phospholipid turnover, working as a substrate for some of cell membranes’ phospholipids, like phosphatidylethanolamine and phosphatidylcholine. These phospholipids participate in lipid signaling pathways and can affect cell proliferation and survival by stimulating membrane receptors or forming second messengers. During the last decade, several in vitro and in vivo studies were conducted to evaluate the antitumor effect of synthetic phosphoethanolamine (Phos-s) on several tumor cell lines. Ferreira et al. showed potent antitumor effects of Phos-s on human breast cancer MCF-7 cells, murine melanoma B16F10 cells, human KS62 and Jurkct leukaemia cells, human melanoma MeWo and SK-MEL-28 cells, human pulmonary H292 cells, Renca and IRPTC cells. Luna et al. showed that encapsulated Phos-s in dioctadecyl(dimethyloammonium chloride (DODAC) liposomes, promote cytotoxicity more selectively and effectively against B16-F10 melanoma and murine hepatocellular carcinoma Hepa1 c1c7 cells. On the other hand, due to the disagreement between researchers about the effectiveness of Phos-s, the Cancer Institute of the State of São Paulo (ICESP), conducted a clinical test with phosphoethanolamine on 72 patients with different cancers. ICESP reported that Phos-s was not effective and only one patient with skin cancer showed slight improvement. In the present study we want to evaluate the antitumor effects of Phosphoethanolamine in an in vivo murine model of Pro-B Acute Lymphoblastic Leukemia (Pro-B ALL) and then investigate its effect on the efficacy of chemotherapeutic agent Vincristine (VCR) in combination therapy.

Methods

Chemicals

Analytical grade phos (Phos-com) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Capsules of synthetic phos (Phos-s) were produced by PDT Pharma (Cravinhos, SP, Brazil) and kindly donated by Dr. Valéria Chierice. Vincristine sulfate (Tecnocris®) was from Zodiac Produtos Farmação (São Paulo, SP, Brazil).

Phos-com and the content of Phos-s capsules (20 mg) were dissolved in 100 µL of a 1:1 sodium bicarbonate 8.4% (w/v): saline 0.9% (w/v) solution. Phos-com is easily soluble while the content of the capsules is partially soluble due to the presence of inorganic phosphates, pyrophosphates and insoluble metal complex of phosphoethanolamine. Phos-s was centrifuged in a 5418 Microcentrifuge (Eppendorf AG, Hamburg, Germany) at 14,000 rpm for 5 min and only the soluble part was used. The final pH was 5 – 6 for Phos-com and 7 – 8 for the soluble part of Phos-s, measured with pH-indicator strips pH 0 – 14 (Merk Millipore, Darmstadt, Germany).
Cell Culture
The RS4;11 cell line (human B-cell leukemia-ATCC CRL-1873) was used in this study. Cells were grown in plastic flasks (25 cm²) containing RPMI 1640 (Sigma) medium supplemented with 10% (v/v) fetal calf serum (Gibco Waltham, MA, USA), 1% penicillin (10000 IU/mL, Gibco), and streptomycin (10mg/mL, Gibco) at 37°C in humidified air with 5% CO2. The medium was changed every 48h.

Xenograft Model of Leukemia
Mice were provided by the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB, State University of Campinas, Sao Paulo, Brazil). All procedures were approved by the institutional animal care committee at UNICAMP (CEUA Protocol number 4336-1), and experiments were performed in accordance with animal care guidelines. Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (NOD.CB17-Prkdcscid/JUnib, The Jackson Laboratory, ME, USA) were housed in sterile conditions using high-efficiency particulate arres- tance filtered micro-isolators and fed with irradiated food and acidified water. Mice were inoculated with RS4;11 cells (c.a. 1x10⁷) via the lateral tail vein, and engraftment was monitored on days 29 and 33 post-infection.

In vivo Experimental Design
Treatment started 33 days after leukemia inoculation. The animals were randomly divided into 3 main groups, each of which was divided into subgroups (5 animals in each sub-group) based on the treatments described in Table 1. Drugs or saline were administered intraperitonially (IP) during working days. Animals from Group 1 received Saline or Vincristine (VCR1) 0.5 mg.Kg⁻¹ or Phos-com (80 or 800 mg.Kg⁻¹) or Phos-s (80 or 800 mg.Kg⁻¹). Based on Lock et al.,[22] this dose of VCR was considered as a therapeutic dose in our protocol. In order to evaluate the effect of phos when co-administrated with VCR, Group 2 received VCR 0.5 mg.Kg⁻¹ associated with Phos-com or Phos-s at 80 or 800 mg.Kg⁻¹. Mice from Group 3 were treated with a sub therapeutic dose of VCR 0.1 mg.Kg⁻¹ alone or in combination with Phos-com or Phos-s 800 mg.Kg⁻¹ to better explore this combination. A more detailed description of the treatments is shown in Table 1.

Apoptosis in vivo and Percentage of Leukemic Cells After Treatment
At the end of treatment, mice were sacrificed by cervical dislocation, and spleen cell suspensions were prepared by mincing the tissues and filtering through 70 µm cell strainers (BD Labware, Franklin Lakes, NJ, USA). Apoptosis was measured by staining cells with Annexin V FITC (BD Pharmi-
gen, San Diego, CA, USA) according to the manufacturer’s protocol as follows: cells were incubated in the stain solution for 15 min at room temperature in the dark. Then 400 µl of binding buffer was added and the cells were analyzed by flow cytometry with FACScalibur (Becton Dickinson). Data were processed using FlowJo software to evaluate the percentage of apoptotic cells. For each analyzed sample, a minimum of 10,000 events were acquired. To measure the percentage of Leukemic cells, 50µL peripheral blood taken from the tail vein was immunostained with anti-CD45 antibody (Invitrogen 11-0459-42). Samples were analyzed by multiparameter flow cytometry on a BD FACSCanto flow cytometer (BD Immunocytometry Systems, San Jose, CA).

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad, San Diego, CA, USA) by one-way analysis of variance (ANOVA) followed by Tukey–Kramer post hoc analysis. Values are presented as mean±standard deviation (SD). Box plots should be interpreted as follows: the middle line is the median; the box extends from the 25th to the 75th percentile; error bars extend down to the lowest value and up to the highest value.

**Mass Spectrometry**

Electrospray mass spectra (ESI-MS) were acquired using a Q Exactive (Thermo Scientific, Bremen, Germany) mass spectrometer with a resolution of 75,000 at m/z 400 and equipped with an electrospray ion source (HESI-II, Thermo). Mass spectra were acquired in the positive ion mode from m/z 60 to 400. The S-lens RF level was set to 10. The spray voltage was +3.5 kV, the heated capillary temperature was 280° C; the sheath gas (N2) flow rate was 2 units and the sample flow rate was 10 µL/min. Collision-induced dissociation (CID) experiments were performed using an isolation window of 2 m/z units and normalized collision energy of 80. In order to have a stable ionic current, it was necessary to reduce the amount of water of injected solutions. They were diluted serially (1:10) twice in a acetonitrile:water:isopropyl alcohol 1:1:0.1 (v/v/v) solution before ESI-MS acquisition. Signals attributed to compounds were accurately assigned with errors less than 2 ppm.

**1H Nuclear Magnetic Resonance (NMR)**

Phos-com and Phos-s injected solutions were also analyzed by 1H NMR using a Bruker Avance III 400 (400 MHz) spectrometer. The chemical shifts (δ) are given in parts per million (ppm). The spectra were referenced against the residual solvent resonance of D2O (δ=4.79). The following abbreviations were used to designate chemical shift multiplicities: appt = apparent triplet, m = multiplet. Phos-com: δ 3.87 (m, 2H), 3.06 (appt, 2H, J = 8 Hz). Phos-s δ 3.97 (m, 4H), 3.82 (m, 2H), 3.66 (appt, 2H, J = 4 Hz), 3.12 (appt, 4H, J = 4 Hz), 3.06 (appt, 2H, J = 4 Hz), 2.97 (appt, 2H, J = 4 Hz).

**Results**

**Phos-com and Phos-s Induce Apoptosis in Human B-Cell Leukemia Infiltrate in Mice Spleen**

The ability of Phos-com and Phos-s to induce apoptosis in human leukemic cancer cells in vivo was evaluated as the percentage of double stained Annexin V (A5) and CD45+ population (Figs. 1c, 1f and 1i) relative to the population of leukemic cells in spleen homogenate obtained from animals of Group 1 (Figs. 1b, 1e and 1h). The entire population of cells (mice plus leukemic clones) of the spleen homogenate can be seen in dot plot gates (Figs. 1a, 1d and 1g). The percentage of A5 stained leukemic cells was approximately 100 times higher for animals treated with 800 mg.Kg⁻¹ Phos-com (Fig. 1f) and 800 mg.Kg⁻¹ Phos-s compared to those treated with saline (Fig. 2).

**Figure 1.** Phos-com and Phos-s induced apoptosis in the human leukemia cell line RS4;11 infiltrated in the spleen of NOD/SCID leukemic mice. Apoptosis was measured by flow cytometry using Annexin V and leukemic clones with anti CD45 antibody. Mice were treated with Saline 0.9% (a–c), Phos-com (800 mg.kg⁻¹, d–f) and Phos-s (800 mg.kg⁻¹, g–i) for 15 days. Total population in spleen homogenate selected in Panels A, D and G, Leukemic and mice cells selected in Panels B, E and H. Apoptotic cells in leukemic population selected in Panels C, F and I.
Phosphoethanolamine Potentiates the Antileukemic Effect of Vincristine in the Peripheral Blood of RS4;11 Engrafted Mice

Initially, phos was tested alone in our pro-B ALL mice model in animals from Group 1. Daily administration of 80 or 800 mg.Kg$^{-1}$ Phos-com or Phos-s for fifteen days was unable to decrease the percentage of leukemic cells in the peripheral blood of RS4;11 engrafted mice in compared to saline group (Fig. 3a). Next, we examined the effect of phos in VCR treated animals from Group 2. We found that concomitant treatment with commercial Phos-com at 800 or even at 80 mg.Kg$^{-1}$ with VCR increased the reduction in the percentage of leukemic cells in the peripheral blood of RS4;11 engrafted mice in compared to saline group (Fig. 3). Phos-s administration, in turn, was able to reduce the percentage of leukemic cells in Vincristine treated animals only when administered at a dose of 800 mg.Kg$^{-1}$ (Fig. 3b).

**Administration of Phos 800 mg.Kg$^{-1}$ Decreases to 1/5 of the Necessary Dose of Vincristine to Achieve Antileukemic Effect in the Pro-B ALL Mice Model**

The synergistic effect of phos on VCR treatment was evaluated more deeply in our pro-B LLA mice model. For this purpose, animals from Group 3 were treated with 0.1 mg.Kg$^{-1}$ VCR alone or in combination with Phos-com or Phos-s at 800 mg.Kg$^{-1}$ (Fig. 4). We first observed that administration of VCR at a subtherapeutic dose (VCR 1/5 0.1 mg.Kg$^{-1}$) was unable to produce the same antileukemic effect observed at 0.5 mg.Kg$^{-1}$. When VCR 0.1 mg.Kg$^{-1}$ was administered with Phos-com at 800 mg.Kg$^{-1}$, this combined treatment was as effective as VRC 0.5 mg.Kg$^{-1}$ treatment in terms of the percentage of leukemic cells in the peripheral blood. A similar effect was not observed when Phos-s was used at 800 mg.Kg$^{-1}$.

**Different Chemical Properties Between Injected Phos-com and Phos-s**

The injected solutions into the engrafted NOD-SCID mice were also analyzed by MS and 1H NMR. The main purpose of these analyses was to determine the chemical species present in basic pH saline solutions of Phos-s and Phos-com that were administered to mice.

ESI-MS of Phos-com and Phos-s solutions (Figs. 5A and C respectively) shows the presence of: [monoethanolamine...
+H]+ of m/z 62.061, [phosphoethanolamine + H]+ of m/z 142.026, [phosphobisethanolamine + H]+ of m/z 185.068 and [dimeric phosphoethanolamine + H]+ of m/z 283.045 (Table 2). The presence of this dimeric specie was confirmed by tandem mass spectrometry. However, 1H NMR analyses of injected solutions, indicates the presence of monoethanolamine and phosphobisethanolamine contaminants only in Phos-s solutions (Fig. 5D) while, as expected, these by-products were not detected in Phos-com (Fig. 5B) demonstrating that the majority of them formed in the ESI font.

**Table 2.** Mass-To-Charge Ratios (m/z) observed in Figure 5.

<table>
<thead>
<tr>
<th>[M + H]+</th>
<th>Observed m/z</th>
</tr>
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<tbody>
<tr>
<td>Monoethanolamine</td>
<td>62.061</td>
</tr>
<tr>
<td>Phosphoethanolamine</td>
<td>142.026</td>
</tr>
<tr>
<td>Phosphobisethanolamine</td>
<td>185.068</td>
</tr>
<tr>
<td>Dimeric phosphoethanolamine</td>
<td>283.045</td>
</tr>
</tbody>
</table>

Figure 4. Synergy of Vincristine in combination with Phos 800 mg.Kg⁻¹ in the Pro-B ALL mice model. Percentage of CD45+ cells (±SD) of RS4;11 engrafted mice after treatment with VCR1 and VCR1/5 (Vincristine 0.1 mg.Kg⁻¹) alone or in combination with Phos-com (800 mg.Kg⁻¹) or Phos-s (800 mg.Kg⁻¹). VCR 1/5 alone was not able to reduce the percentage of leukemia when compared to saline treatment (65.04±21.06 and 77.04±9.56 respectively). VCR 1/5 combined with Phos-com decreased leukemia to 36.28±10.05 very similar to what was observed at the therapeutic dose of VCR1 (33.5±12.67). A similar effect was not observed when Phos-s was used (X±Y). **p<0.005, ***p<0.001.

Figure 5. Chemical characteristics of injected solutions. Representative ESI(+)MS of Phos-com (a) and Phos-s (b). Color bars were added to facilitate visualization of ion peaks of interest: monoethanolamine (m/z 62.061, [M+H]+), phosphoethanolamine (m/z 142.026, [M+H]+), phosphobisethanolamine (m/z 185.068, [M+H]+), dimeric phosphoethanolamine (m/z 283.045, [M+H]+). 1H NMR (400 MHz, D₂O) of Phos-com (a) and Phos-s (b) solutions. Signals of methylene hydrogen atoms (CH₂) (Fig. 1) from P = phosphoethanolamine, M = monoethanolamine and B = phosphobisethanolamine.
Discussion

Previous in vivo and in vitro studies\textsuperscript{[14, 16–20]} have indicated the ability of Phos-s to induce apoptosis and its antitumor effect in various cancer cells. In this study, we used commercial phos (Phos-com/ Sigma Aldrich) and the content of Phos-s capsules provided by PDT Pharma that produced according to the in-house methodology of Institute of Chemistry, USP-SC, to test the pro-apoptotic and antileukemic effect of phos in a murine model of Pro-B LLA, and then investigated its effect on the efficacy of vincristine (VCR) in combination therapy.

The pro-apoptotic of phos is clearly visible in our model since both Phos-com and Phos-s were able to increase the percentage of apoptotic leukemic clones sequestered by the spleen (Figs. 1F, 11 and 2). This is in agreement with the results of Ferreira et al.\textsuperscript{[14] They showed phos induces apoptosis in vitro to leukemia cell lines and also induces apoptosis of immature cells in the spleen and liver in vivo mice model of promyelocytic leukemia (APL).\textsuperscript{[14]} However, as shown in Figure 2A, contrary to previous studies\textsuperscript{[14, 16–20]}, neither Phos-com nor Phos-s alone showed antitumor effect and was not able to reduce the percentage of leukemic cells in vivo in the peripheral blood of transplanted mice.

Pro-B ALL is one of the leukemias with the highest recurrence rate\textsuperscript{[15]}, and considering that residual cancer cells in the affected organs are the main cause of recurrence of this cancer, we tested a combined treatment protocol using VCR and Phos-com or Phos-s. We found that VCR + Phos-com combination treatment was able to more effectively reduce the percentage of leukemic clones even at a lower dose of 80 mg.Kg\textsuperscript{-1}. For Phos-s, this increase in the therapeutic profile of VCR treatment was only observed when a dose of 800 mg.Kg\textsuperscript{-1} was used (Fig. 3B).

Our administration of subtherapeutic treatment with VCR (0.1 mg.Kg\textsuperscript{-1}) in combination with Phos-com 800 mg.Kg\textsuperscript{-1} which was able to restore the efficacy of VCR in reducing the percentage of the leukemic clones, confirms the synergy between phos and VCR (Fig. 4). We do not know if there are clinical advantages when we decrease the dose of chemotherapy associated with phos, but probably toxicity of chemotherapy will be lower. Since no deleterious effects of phos administration i.e. loss of body weight or increase in mortality rates were observed, co-administration of phos with a subtherapeutic dose of VCR in our in vivo model shows that it is beneficial in Pro-B ALL treatment.

We next addressed, from a chemical point of view, the qualitative differences between these substances that may explain the different responses to our in vivo leukemia model. MS analysis of the injected solutions shows many more similarities than differences (Figs. 5A and 5C). The presence of the contaminants monoethanolamine and phosphobisethanolamine in Phos-com, may be explained by source fragmentation and side reactions. Dimeric phos is likely to be residual from the intermolecular hydrogen bonds observed in phos crystals and present in both solutions. The two hydroxyls and amino groups can produce different species in solution depending on the pH, which may eventually lead to the formation of monoethanolamine and phosphobisethanolamine contaminants. Our 1H NMR analysis confirmed the presence of contaminant species of monoethanolamine and phosphobisethanolamine only in Phos-s capsules. Our data also confirmed the chemical stability of Phos-com under our experimental conditions, and the presence of contaminant monoethanolamine and phosphobisethanolamine originated mainly from the synthetic procedure of Phos-s.

In this study we aimed to investigate the effects of Phos-s capsules consumption and compare it with the putative consumption of the same amount of phos from a commercial source. That’s why, no previous adjustments were made in phos contents of both capsules. Previous analysis of the soluble content of Phos-s capsules indicated the presence of 45% phos, 32% monoethanolamine and 7% fosfobisethanolamine. However, the differences observed in the performance of Phos-s and Phos-com may be explained by differences in the levels of phos present in the injected solutions, and it also concludes that a possible therapeutic effect of Phos-s capsules may ultimately be attributed to its phos content.

Conclusion

Our results showed that although phos were able to increase the percentage of apoptotic leukemic clones sequestered by the spleen, was not effective in reducing ProB ALL leukemic clones in the in vivo mouse model, however, it was able to be used in combination with VCR without harming the mice and increased the antitumor efficacy of VCR even at reduced dose.

Disclosures

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Ethics Committee Approval: All procedures were approved by the institutional animal care committee at UNICAMP (CEUA Protocol number 4336-1).

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

References

Supplementary Figure 1. Using flow cytometry to detect apoptotic cells collected from NOD/SCID mice's spleens obtained after 14 days of treatment and labeled with human CD45.

Supplementary Figure 2. The glass capillary tube is placed next to the eye at an angle of 45° to the sagittal and coronal planes. The capillary tube rotates with pressure on the orbital bone just in front of the zygomatic arch until blood flows from the orbital sinus into the superficial temporal vein. The capillary tube moves back millimeters and blood is collected by capillary action. The entire procedure is carried out by a specialized technician.