# Antitumor properties of a new non-anticoagulant heparin analog from the mollusk Nodipecten nodosus: effect on P-selectin, heparanase, metastasis and cellular recruitment

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# Antitumor properties of a new non-anticoagulant heparin analog from the mollusk *Nodipecten nodosus:* effect on P-selectin, heparanase, metastasis and cellular recruitment

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## Abstract

**Inflammation and cancer are related pathologies acting synergistically to promote tumor progression. In both, hematogenous metastasis and inflammation, P-selectin participates in interactions involving tumor cells, platelets, leukocytes and endothelium. Heparin has been shown to inhibit P-selectin and as a consequence it blunts metastasis and inflammation. Some heparin analogs obtained from marine invertebrates are P-selectin inhibitors and do not induce bleeding effects. The present work focuses on the P-selectin blocking activity of a unique heparan sulfate (HS) from the bivalve mollusk Nodipecten nodosus. Initially, we showed that the mollusk HS inhibited LS180 colon carcinoma cell adhesion to immobilized P-selectin in a dose-dependent manner. In addition, we demonstrated that this glycan attenuates leukocyte rolling on activated endothelium and inflammatory cell recruitment in thioglycollate-induced peritonitis in mice. Biochemical analysis indicated that the invertebrate glycan also inhibits heparanase, a key player in cell invasion and metastasis. Experimental metastasis of**

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## Introduction

The relationship between cancer and inflammation has been increasingly reported during the last decade [1]. However the connection between these two pathologies was first suggested about 150 years ago [2]. Whereas acute inflammation is part of the organism defense response, chronic inflammation can lead to cancer. Indeed, patients with ulcerative colitis and Crohn's disease, have higher risk to develop colorectal cancer [3]. On the other hand, during hematogenous metastasis, natural killer cells can attack tumor cells, decreasing the metastasis rate. Thus, the function of leukocytes in tumor biology is complex [4] and represents an attractive research.

Hematogenous metastasis and inflammatory cell recruitment strongly depend on selectin function. P-selectin is a family member of glycan-recognizing adhesion molecules. In endothelial cells, P-selectin is stored in Weibel–Palade bodies, whereas in platelets, it occurs in α-granules. P-selectin is readily exposed at the surface of platelets upon activation, mediating its interaction with leukocytes and endothelial cells [10]. Tumor cells are characterized by aberrant glycosylation patterns [11, 12], including the over-expression of highly branched or sialylated oligosaccharides, especially fucosylated glycans, such as sialyl-Lewis$^{X}$ and sialyl-Lewis$^{a}$. These glycans are ligands for selectins and their presence is related to poor prognosis due to increased metastatic disease [13, 14].

The metastatic process is comprised of several steps that include degradation of basement membrane, entry of cancer cell into the bloodstream, evasion of innate immune surveillance, adhesion to the vascular endothelium of secondary sites with subsequent extravasation and colonization. Once in the bloodstream, cancer cells are covered by platelets, in a P-selectin-dependent process, forming a natural barrier against immune system cells [6]. On the other hand, leukocytes recruitment during inflammation is mediated by P- and L-selectins. These adhesion molecules are involved in the first steps of cellular

recruitment by decreasing the rolling velocities of leukocytes, contributing to their adhesion and arrest at sites of inflammation.

Cancer patients have high-risk of developing thromboembolic disease and therefore, heparin has been used as a prophylactic therapeutic agent. Retrospective analysis of patients under heparin therapy revealed a better prognosis of the malignant disease, which has not been associated with the anticoagulant effect of the drug [15, 16]. Several studies aimed to investigate the anti-cancer effect of heparin revealed that it attenuates experimental metastasis in animals [9], mainly by binding to P-selectin and decreasing the interaction between tumor cells and platelets. Additionally, heparin can also attenuate metastasis by inhibition of heparanase [17], the only known mammalian endoglycosidase that cleaves heparan sulfate and is over-expressed in essentially all human tumors. In fact, heparanase over-expression correlates with poor prognosis in a variety of cancers [18, 19].

We have previously shown that heparin analogues obtained from marine invertebrate bind to P-selectin, attenuating metastasis, inflammation and thrombosis [20, 21]. In a recent study, we described a unique heparan sulfate (HS) isolated from the bivalve mollusk *Nodipecten nodosus.* This sulfated polysaccharide is formed by glucuronic acid and glucosamine and can also contain a rare pattern of sulfation on carbon 2 and 3 of the glucuronic acid units. We also described that this compound was able to inhibit thrombus growth without inducing bleeding effect [22].

Although increasing evidence points to a beneficial therapeutic action of heparin in cancer patients, its bleeding effect still limits its use. Here, we addressed the ability of HS from *N. nodosus* to attenuate leukocyte recruitment and cancer metastasis. Our results reveal that the mollusk HS presents effective P-selectin inhibitory activity, decreasing the binding of carcinoma cells to P-selectin *in vitro* and its interaction with platelets *in vivo*. The mollusk glycan also inhibits heparanase enzymatic activity *in vitro*. Moreover, we have demonstrated that this glycan attenuates hematogenous metastasis and polymorphonuclear cells recruitment *in vivo*.

## Materials and methods

### *Cell lines and reagents*

Human colon carcinoma cells (LS180; ATCC, Manassas, VA, USA) were grown in minimum essential medium-α (MEM-α) (Invitrogen,Carlsbad, CA, USA) supplemented with

10% fetal bovine serum (FBS) (Invitrogen). Mouse Lewis lung carcinoma cells (LLC) were grown in Dulbecco's modified Eagle's medium (DMEM) (Vitrocell) supplemented with 10% FBS. All reagents were from Sigma (St Louis, MO, USA) unless otherwise stated. UFH (Liquemine) was obtained from RochePharma (Reinach, Switzerland).

*Isolation of HS from N. nodosus*

Adult specimens of the bivalve mollusk *Nodipecten nodosus* (Linnaeus, 1778) were collected from Baia da Ilha Grande, Angra dos Reis, Rio de Janeiro, Brazil. The polysaccharides were extracted by protease digestion and purification as described previously [22].

*Inhibition of tumor cell binding to immobilized p-selectin*

The ability of glycosaminoglycans to inhibit the adhesion of calcein AM-labeled LS180 cells to immobilized P-selectin chimeras was performed as described previously [23]. Each glycan was tested in triplicate wells at each concentration.

*Intravital microscopy*

Leukocyte rolling in the mesenteric venules was analyzed after 4 hours of lipopolysaccharide (LPS) (Sigma; 0,5mg/kg) or PBS intravenous injection. Adult male and female Wistar rats (250 g body weight) were anesthetized with an intramuscular injection of 100 mg kg-1 of ketamine (Cristália, São Paulo, Brazil) and 16 mg kg-1 of xylazine (Bayer AS, São Paulo, Brazil). An abdominal midline incision (around 1,5 cm) was performed and the mesentery was exposed for analysis. After positioning under the microscope, a 30 min equilibration period preceded quantitative measurements. The microscopy used was Zeiss Axio ImagerA1. Leukocyte rolling was counted for 10 min. In the group that received LPS, leukocyte rolling was evaluated before and after circulation of *N. nodosus* HS for 5 min (2mg/kg - intravenous injection). Videos are available at supplemental material.

*Thioglycollate-induced peritoneal inflammation*

Mice were injected intraperitonealy with 4% thioglycollate (1mL). After 5 min, *N. nodosus* HS was administrated via tail vein injection. Mice were sacrificed after 3 hours and peritoneal lavage was collected using 4 mL ice-cold PBS, containing 3 mM EDTA to prevent clotting. The peritoneal fluid, 200 µL, was analyzed after cytospin preparation by hematoxylin and eosin staining. Then, differential counting was performed to evaluate the amount of polymorphonuclear cells present in the peritoneal cavity. P-selectin-deficient mice (P-sel -/-) and wild type mice were used in this experiment.

*Tumor cell-platelets association in vivo*

The formation of tumor cell-platelet complex was performed as described before [9]. Briefly, LLC cells were harvested with 2 mM EDTA in phosphate-buffered saline (PBS), labeled with calcein AM, and injected intravenously into mice with or without previous intravenous application of 200 µg of *N.nodosus* HS . 30 min later lungs were harvested for analysis. Lung sections were stained with goat anti-integrin αIIb (CD41) (Santa Cruz biotechnology-sc6602), followed by anti-goat Cy3-conjugated antibody (Sigma), and analyzed by immunofluorescence microscopy. The extent of platelet association with tumor cells was quantified by evaluating calcein-labeled cells present in 40 fields of lung sections.

*Heparanase enzymatic activity assay*

Preparation of sulfate-labeled extracellular matrix (ECM)–coated dishes and determination of heparanase enzymatic activity were performed as previously described [24, 25]. Briefly, sulfate-labeled ECM coating the surface of 35-mm culture dishes was incubated (4 hours, 37°C, pH 6.0) with constitutively active recombinant human heparanase (120 ng/ml) in the absence or presence of 5 µg/ml of mollusk HS, as described [18]. The incubation medium containing sulfate-labeled degradation fragments was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 ml) were eluted with PBS and their radioactivity counted in a β-scintillation counter. HS degradation fragments were eluted at $0.5 < K_{av} < 0.8$ (peak II, fractions 10–25).

*Experimental metastasis model*

Mice (8 - 10 weeks old) were intravenously injected with $10^6$ LLC cells via the tail vein. *N. nodosus* HS or PBS administration was performed 10 min prior to cell injection. Mice were sacrificed after 21 days. The lungs were macroscopically evaluated for the number of metastatic foci.

**Results**

*HS from N.nodosus attenuates tumor cell binding to P-selectin*

Because P-selectin is a crucial mediator of cell-cell interactions during inflammation and cancer, we evaluated whether HS from *N.nodosus* could inhibit P-selectin. For this purpose, we analyzed the ability of this compound to impair adhesion of LS180 cells to immobilized P-selectin. It is known from previous work that this colon cancer cell line express high content of selectin ligands [26]. *N.nodosus* HS decreased tumor cell binding to P-selectin in a dose dependent manner, yielding an $IC_{50}$ of 38,19 µg/mL, comparable to that of unfractionated heparin from porcine intestine (UFH) ($IC_{50}$ of 24,51 µg/mL) (Figure 2). It is worth pointing that because UFH presents lower molecular weight (around 15 kDa) than HS from *N.nodosus* (around 30 kDa), the molar concentration of UFH is higher than that of HS from *N.nodosus.*

*HS from N.nodosus reduces leukocyte rolling and cell recruitment after inflammatory stimulus*

While the involvement of inflammatory cells during several steps of cancer progression is well documented, little is known about the underlying mechanism. Because leukocyte rolling on activated endothelium during inflammatory response is mediated by P-selectin we investigated how leukocyte rolling is affected by *N.nodosus* HS treatment. Previous studies have shown that LPS treatment induces P-selectin upregulation in endothelial cells [27]. Based on this, we analyzed leukocyte rolling on LPS-activated endothelium before and after *N.nodosus* HS administration, applying intravital microscopy. It was observed that *N.nododus* HS treatment inhibited LPS-induced leukocyte rolling to the basal level (Fig. 3, A). Because leukocyte rolling is the first step for cell recruitment, we further evaluated the effect *N. nodosus* HS on a thioglycollate-induced peritonitis model in mice. After 3 hours of inflammatory stimulus, the peritoneal lavage was harvested and differential counting of leukocytes was performed. Figure 3B shows that thioglycollate

promoted a significant increase in the recruitment of polymorphonuclear cells to the peritoneal cavity, compared to control. *N. nodosus* HS treatment resulted in a 70% reduction in leukocyte recruitment. This effect was similar to observed reduction of leukocyte recruitment in untreated P-selectin-deficient mice (P-sel$^{-/-}$). Interestingly, treatment of P-sel$^{-/-}$ mice with HS from *N. nodosus* led to a lower leukocyte recruitment in comparison to both HS-treated wild-type or untreated P-sel$^{-/-}$ mice (Figure 3C). These findings suggest an additional mechanism involved in the inhibition of leukocyte recruitment by *N. nodosus* HS besides P-selectin. Considering that previous studies have shown that some glycosaminoglycans also inhibit L-selectin [21], we suggest that this glycan may additionally be blocking L-selectin.

*N. nodosus HS inhibits association between tumor cells and platelets in vivo*

As mentioned before, P-selectin dependent leukocyte recruitment and intercellular interactions are crucial during inflammation and tumor metastasis as well. P-selectin-mediated platelet association with tumor cells contributes to the accomplishment of metastasis. In previous studies, we showed that glycosaminoglycans from marine invertebrates could disrupt this association. In order to analyze the ability of HS from *N. nodosus* to inhibit tumor cell-platelet interactions *in vivo*, HS (200 µg/mouse) was injected via tail vein 5-10 min before injection of LLC cells. Lungs were analyzed by immunofluorescence 30 min after tumor cell (calcein labelled) injection. While in the control group, approximately 70% of tumor cells were associated with platelets (Fig. 4), *N. nodosus* HS administration significantly reduced tumor cell-platelet association to 30%.

*Mollusk HS inhibits heparanase and attenuates experimental metastasis*

LLC cells express high amounts of heparanase [28], which plays a relevant role in tumor invasion and metastasis [29]. Because heparanase has been pointed as a molecular target of heparin in cancer, we wondered whether HS from *N. nodosus* inhibits heparanase enzymatic activity, possibly affecting tumor invasion. For this purpose, we used a naturally produced sulfate-labeled ECM as substrate and measured the release of HS degradation fragments upon incubation with heparanase [24, 25]. As demonstrated in Figure 5, the mollusk HS inhibited heparanase enzymatic activity. Because metastasis efficiency depends on P-selectin and heparanase, and UFH treatment is known to decrease tumor metastasis, we sought to assess the anti-metastatic activity of *N. nodosus* HS [6, 9]. In order to investigate

this, we applied an experimental metastasis model in mice, using Lewis lung carcinoma (LLC) cells that express P-selectin ligands and heparanase. This experiment involved administration of 200 µg *N. nodosus* HS followed by injection (tail vein) of LLC carcinoma cells. Figure 6 shows the effect of the mollusk HS treatment on metastasis. Whereas the number of metastatic foci was high in control lungs, HS-treated animals presented just few metastatic foci. We also noticed that the tumor burden in control lungs was higher than in the treated animals. Overall, these results indicate that the mollusk HS may be an attractive therapeutic drug to block both P-selectin-mediated interactions and heparanase activity, blunting metastasis and inflammation without inducing bleeding effects.

**Discussion**

Cell interactions among leukocytes, platelets and endothelium are mediated by selectins and contribute to the pathophysiology of inflammation and metastasis. Several studies have shown that heparin can block these interactions and therefore attenuate hematogenous metastasis and inflammatory cell recruitment. In 2010, we described a unique heparan sulfate obtained from the bivalve mollusk *Nodipecten nodosus,* which presents 2 or 3 O-sulfation on glucuronic acid (Fig. 1). This compound is extracted from organs commonly discarded during preparation for commercialization and presents anti-thrombotic activity without inducing any bleeding effect [22]. Therefore, we thought to investigate if this glycan could be a useful p-selectin inhibitor. In order to evaluate this inhibitory potential we performed *in vitro* and *in vivo* experiments and showed that the mollusk HS inhibits p-selectin interaction with colon carcinoma cell line (LS180), decreases cell rolling and inflammatory cell recruitment. Additionally it attenuates platelets-tumor cell association and heparanase enzymatic activity, thereby blunting metastasis of Lewis lung carcinoma cells (LLC).

Leukocyte function in tumor biology has deserved attention in cancer research [30, 31]. It has been proposed that, in some types of cancer, the inflammatory cascade is already activated before the tumor initiates [3]. Conversely, in other situations the malignant microenvironment promotes inflammatory cells recruitment, which in turn can induce tumor growth [32, 33] or lysis of tumor cells. Inflammatory leukocyte rolling on activated endothelium is a p-selectin mediated event. Applying intravital microscopy, we showed that mollusk HS decreases leukocyte rolling after endothelial cell activation (Fig. 3A). We speculate that as a consequence of this inhibition polymorphonuclear cells recruitment to the

peritoneal cavity was attenuated as well (Fig.3B). We also observed a decrease in leukocyte recruitment in p-selectin deficient mice (Fig.3C). As some glycosaminoglycans also bind to L-selectin, inhibition of leukocyte recruitment through L-selectin interaction can be expected, as well. Therefore we suggest that HS from *N. nodosus* might also inhibit L-selectin.

Another branch of p-selectin-mediated interactions occurs during dissemination of metastatic cancer cells. Along this process after bloodstream entry, tumor cells are often covered with platelets in a p-selectin dependent manner. This interaction confers the tumor cells with physical shielding mediated by platelets, avoiding natural killer cell mediated tumor cell lysis. We found that the mollusk glycan inhibited significantly tumor-cell platelet association, already 30 min after tumor cells injection (Fig. 5). We suggest that without this interaction tumor cells would be more vulnerable to immune surveillance and as a result the metastasis rate would decrease. Using LLC cells, which are carcinoma cells that express selectin ligands at the cell surface [34], we demonstrated that the mollusk glycan drastically attenuates seeding of tumor cells to the lungs. An elegant work of Labelle et al (2011) showed that apart of protection from leukocytes, platelets can induce epithelial-mesenchymal transition in tumor cells via TGFβ mediated-mechanism and thereby promote metastasis. Similarly, through inhibition of platelet-tumor cell interaction the mollusk glycan may exert anti-metastatic effect by suppressing the epithelial mesenchymal transition induced by platelets.

When tumor cells exit the bloodstream they need to degrade the sub-endothelial basement membrane, which is rich in heparan sulfate, in order to colonize a secondary site [35]. Previous studies have shown that heparin-mimicking compounds attenuate metastasis by acting as heparanase inhibitors as well [36]. Therefore we suggest that the heparanase inhibitory activity of the mollusk glycan contributes to the anti-metastatic effect observed in our experiments.

Overall, the present work identifies a new p-selectin inhibitor that does not induce hemorrhagic effects observed in mammalian heparin. We also showed that this compound attenuates thrombosis, inflammation and metastasis. Since cancer disease is usually associated with thrombotic events we suggest that the mollusk HS is an attractive candidate to be a therapeutic drug for cancer-associated thrombosis and cancer-related inflammation.

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**Figure Legends**

Figure 1. **Structure of HS from N. nodosus.** The residues denoted with G and A are glucoronate and D-glucosamine, respectively.

Figure 2. **HS from N.nodosus attenuates p-selectin binding to LS180 tumor cells**. LS-180 human colon carcinoma cells adhesion to immobilized P-selectin chimera was measured in the presence of increasing concentrations of HS from N. nodosus (open circles) or unfractionated heparin (filled squares). The curves are representative of three independent experiments. See "Material and methods" for experimental details.

Figure 3. **HS from N. nodusus inhibitis leukocyte rolling and polymorphonuclear cell recruitment.** (A) Leukocyte rolling (cells/min) along the endothelium of post-capillary venules in distal ileum was measured by use of intravital microscopy. Four hours before counting, rats were intravenously injected with LPS (0,5 mg/Kg) or saline. In rats treated with LPS the leukocyte rolling was measured before and after intravenous administration of HS from N. nodusus (2 mg/Kg). (B-C) Thioglycolate was injected intraperitonealy, followed by intravenous injection of mollusk HS (0.5 mg/mouse) 5 minutes later. Peritoneal lavage was harvested 3 hours later. Cells were stained with hematoxylin and eosin and differentially counted for mononuclear and polymorphonuclear cells. (B) wild- type mice. (C) P-selectin knockout mice. Arrows indicate polymorphonuclear cells. Scale Bar represents 20 μm. The statistical significance was determined by one-way ANOVA (*** $p < 0.001$).

Figure 4. **HS from N. nodosus inhibits platelet adhesion to tumor cell *in vivo*.** LLC cells labeled with calcein AM were intravenously injected via tail vein. (A) Representative images of platelet-tumor cell association in lungs of mice injected with either HS from N.nodosus (200μg) or PBS euthanized 30 min later. Scale bar represent 20 μm; (B) Numbers of platelet–tumor cell aggregates are presented as percentages (in columns) of all counted tumor cells. The statistical significance of tumor cell–platelet association was determined by Test-T (*** $p < 0.001$).

Figure 5. **HS from N. nodosus is a heparanase inhibitor.** The ability of mollusk HS to inhibit recombinant heparanase enzymatic activity was determined as described in Materials and Methods. Data are representative of three independent experiments.

Figure 6. **Experimental lung metastasis of LLC carcinoma cells is attenuated by mollusk HS treatment.** Mice were intravenously injected with 200 μg of mollusk HS, 10 minutes before injection of $10^6$ LLC cells and killed 21 days later. Metastatic foci in the lungs were evaluated macroscopically. (A) Representative images of lungs harvested from mice injected with mollusk HS versus control. (B) Counting of metastatic foci.

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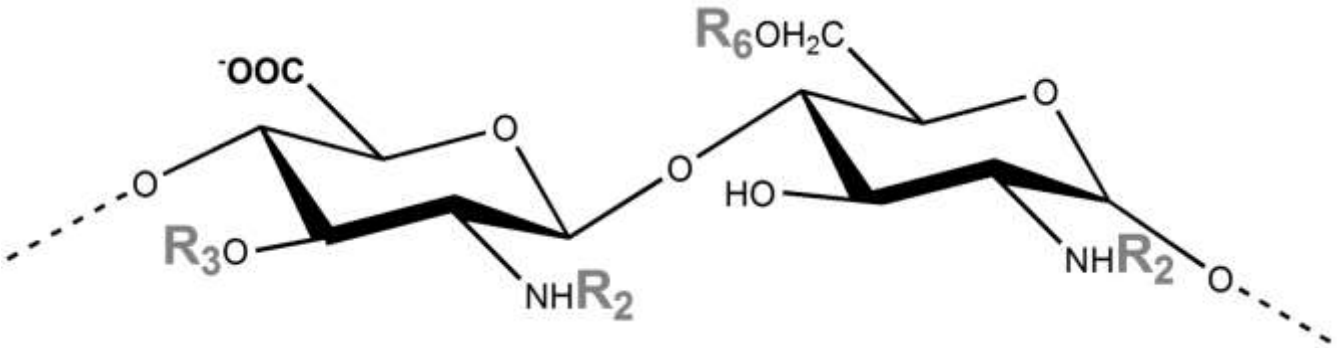
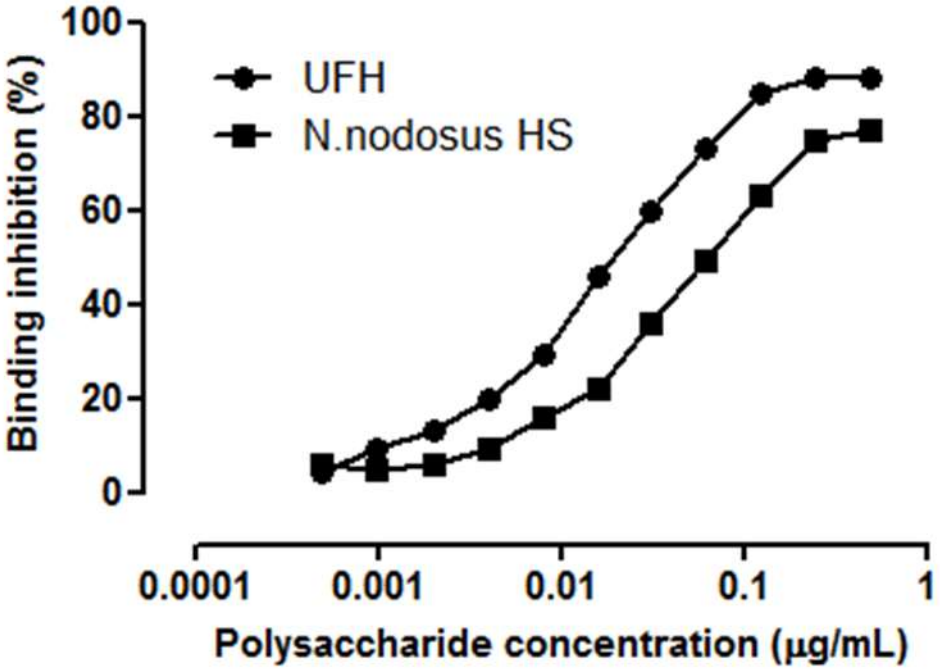



-4-Glucuronate-β1 → 4-glucosamine-α1-

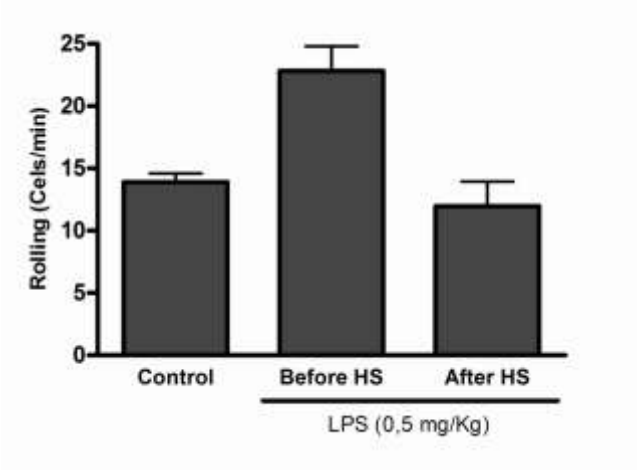
| $R_2$ | $R_3$ | $R_2$ | $R_6$ |
|---|---|---|---|
| *H:* ***71.6%*** | *H:* ***79.2%*** | *Ac:* ***61%*** | *H:* ***84%*** |
| $SO^{3-}$: ***28.4%*** | $SO^{3-}$: ***20.8%*** | $SO^{3-}$: ***39%*** | $SO^{3-}$: ***16%*** |

Gomes AM *et al.*, Figure 2

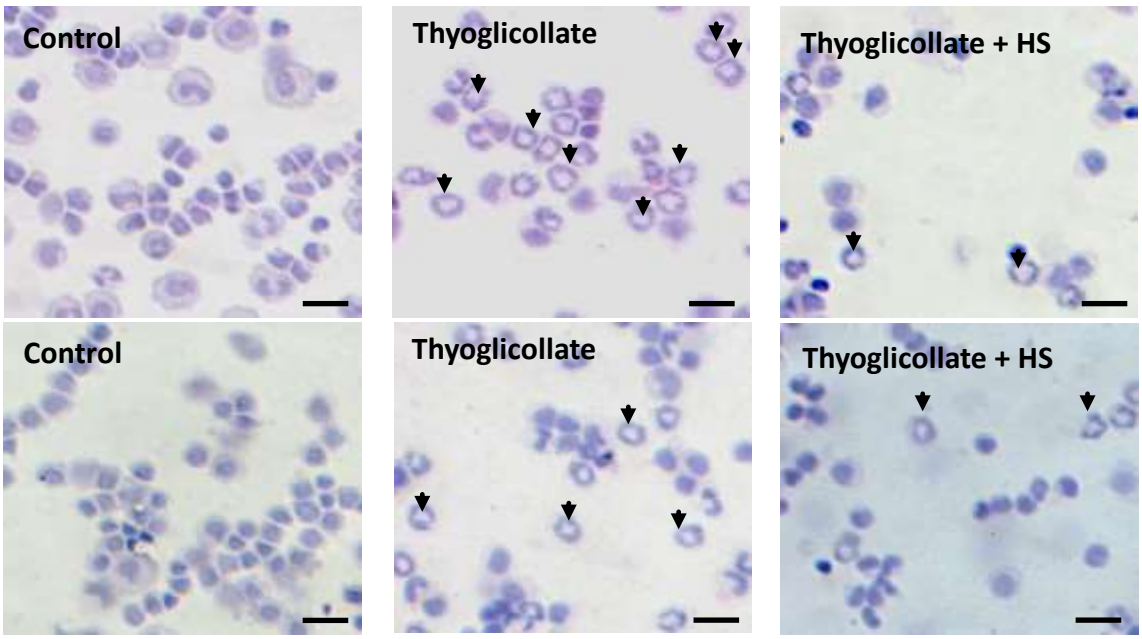



Gomes AM *et al.*, Figure 2

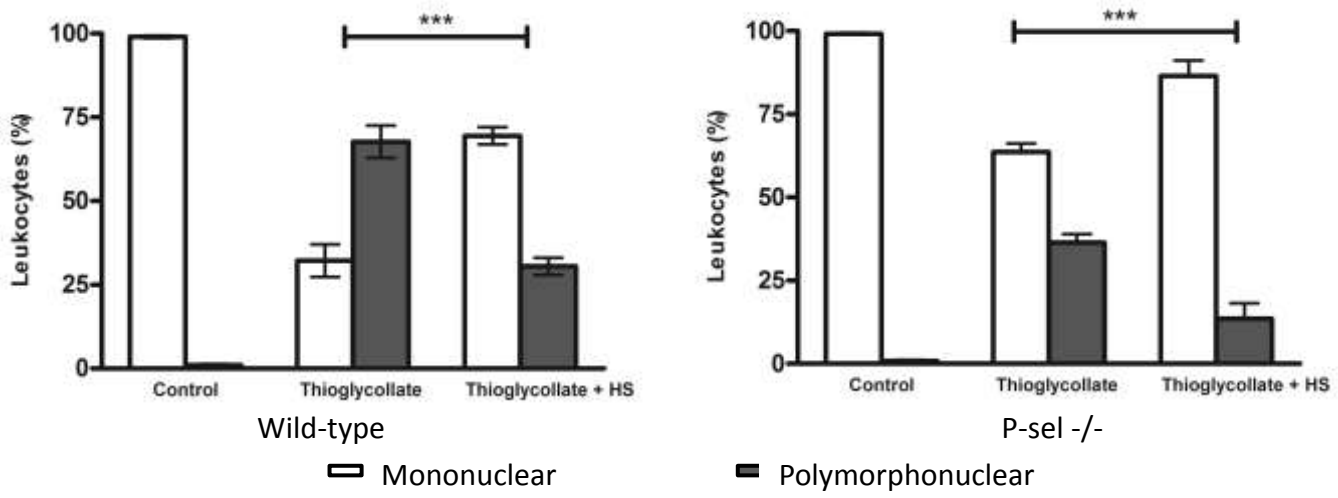
A




B

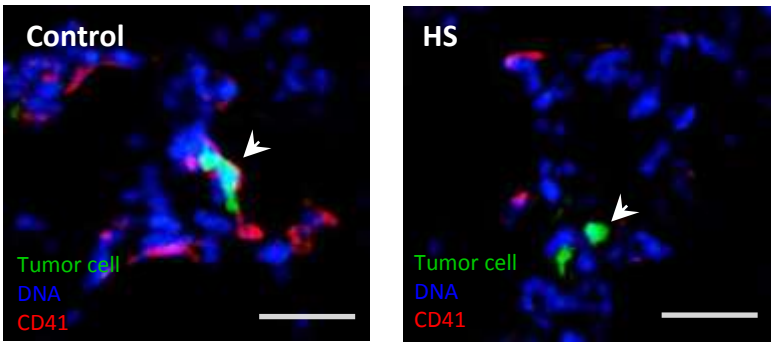



C

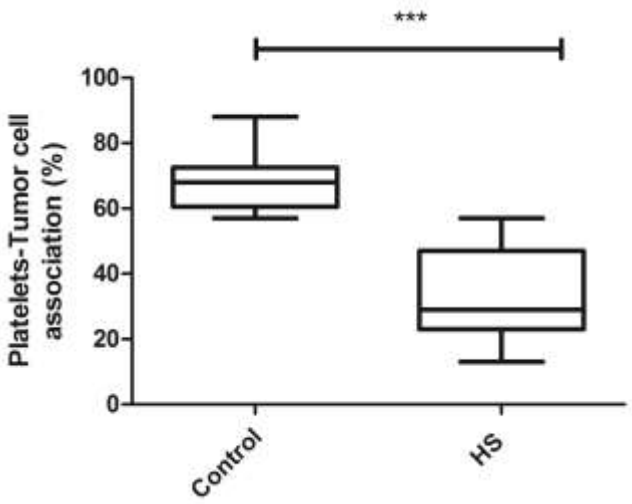



Gomes AM *et al.*, Figure 3

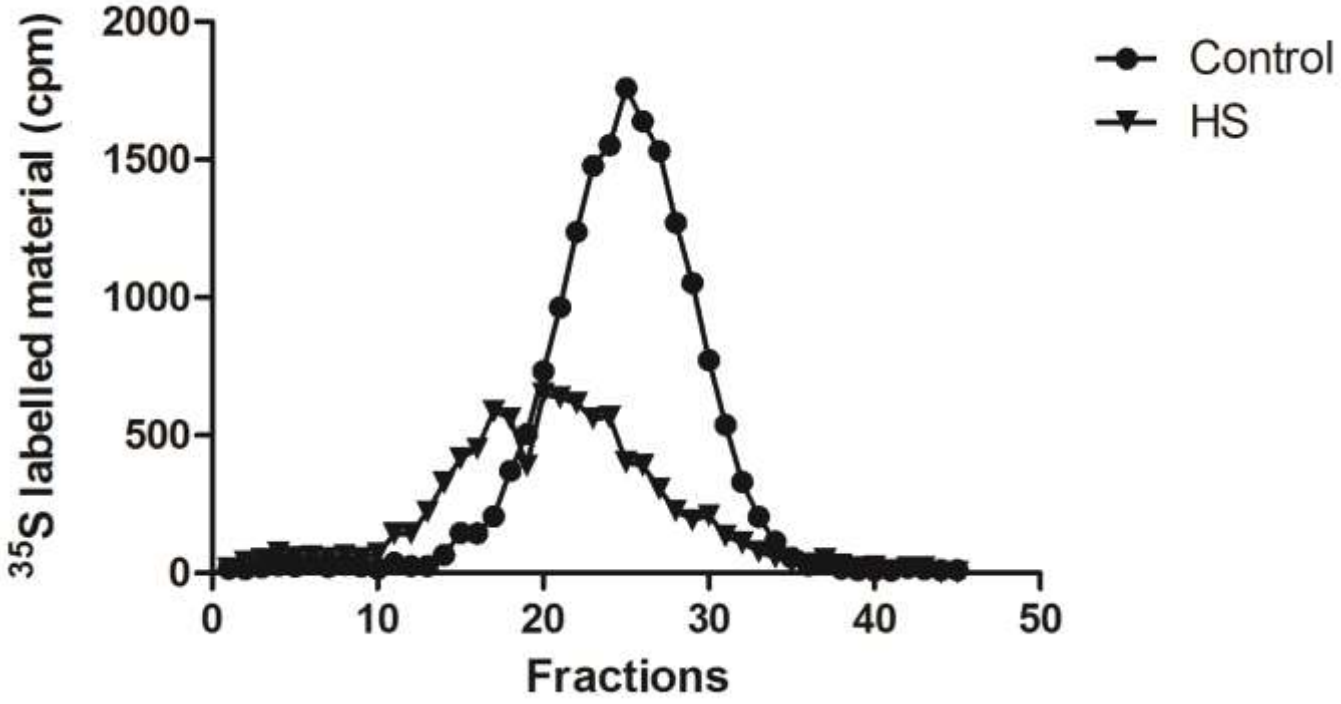
A




B

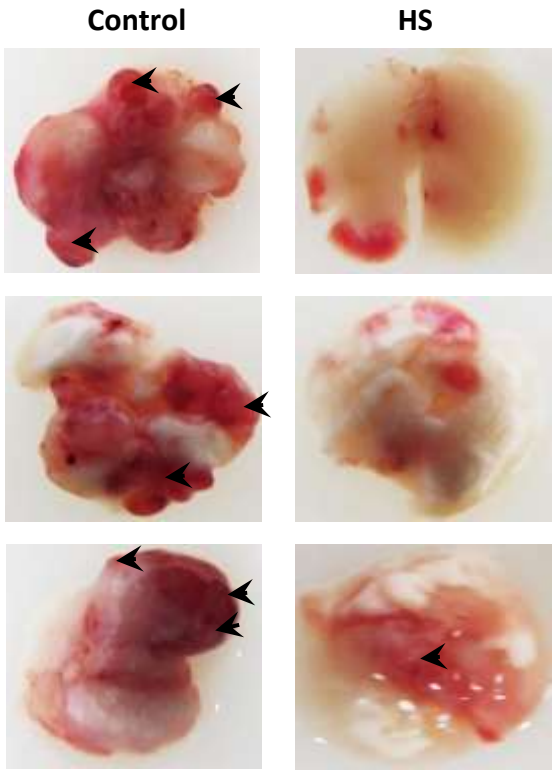



Gomes AM *et al.*, Figure 4




Gomes AM *et al.*, Figure 5

A

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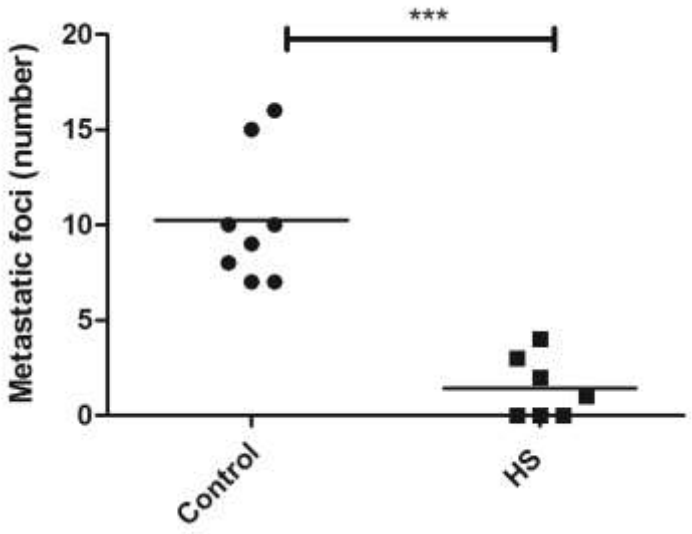


Gomes AM *et al.*, Figure 6