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Association of α_2-HS Glycoprotein with Neurogenic Heterotopic Ossification in Patients with Spinal Cord Injury

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: The aim of this study was to explore the relationship between the α_2-HS glycoprotein concentrations in serum and the occurrence of neurogenic heterotopic ossification (NHO) in patients with spinal cord injury (SCI).

Material/Methods: During the period between January 2011 and January 2012, 75 patients (67 male) with paraplegia caused by spinal cord injury were enrolled. The patients were divided into 2 groups in accordance with the occurrence of heterotopic ossification based on the results high-frequency ultrasound on the bilateral hip joint. The levels of α_2-HS glycoprotein, C-reactive protein (CRP), D-dimer, and bone morphogenetic protein (BMP) were detected by ELISA.

Results: We found a significant decrease of α_2-HS glycoprotein in SCI patients with NHO compared to SCI patients without NHO. In contrast, a significant elevation of serum calcium, D-dimer, BMP, and CRP was observed in SCI patients with NHO. The degree of maturity of NHO did not influence the level of α_2-HS glycoprotein. Multivariate liner regression analysis showed that the level of serum α_2-HS glycoprotein was correlated with CRP and spasticity.

Conclusions: The decreased level of α_2-HS glycoprotein may be related to the formation of neurogenic heterotopic ossification in patients with spinal cord injury. Our results suggest that α_2-HS glycoprotein might be a risk factor for NHO in patients with SCI.

MeSH Keywords: Activities of Daily Living • Inflammation • Neurology

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Background

Neurogenic heterotopic ossification (NHO) is the abnormal formation of mature lamellar bone where bone does not normally exist, usually in the soft tissues [1]. It is one of most common complications in patients with central nervous system disorders such as spinal cord injury (SCI). Severe heterotopic ossification might limit the degree of joint movement, and even cause ankylosis, reducing the ability to perform activities of daily living and affecting the recovery of patients [2,3]. There is no effective treatment for neurogenic heterotopic ossification, and the early detection of NHO is crucial for early treatment and good prognosis. Risk factors such as edema, prolonged swelling and demographic factors (e.g., age and sex) have been reported to be associated with increased risk of NHO [2,4–6]. Spasticity, pressure ulcers, and urinary tract infections have also been suggested to be risk factors for NHO formation [7]. Although many risk factors have been identified for the formation of NHO, most do not have clear biological basis and the association with NHO is relatively weak.

The α_2-HS glycoprotein, also referred to as human fetuin A (Fetuin A of α2 Heremans-Schmid glycoprotein, AHSG), is a plasma glycoprotein belonging to the fetuin family [8]. It serves as an extracellular calcium-regulatory protein, which inhibits Ca×PO4 precipitation [9–12]. The α_2-HS glycoprotein is abundant in the extracellular space which is responsible for over half of the precipitation inhibitory effect of serum [8,11]. It can also markedly inhibit ectopic calcification [13]. To search for risk factors with a relatively clear biological basis, we hypothesized that α_2-HS glycoprotein downregulation might be associated with the occurrence of neurogenic heterotopic ossification. Therefore, we analyzed the α_2-HS glycoprotein level in SCI patients with or without hip heterotopic ossification in this study.

Material and Methods

Study design

From January 2011 to January 2012, 75 patients (67 male) with paraplegia caused by spinal cord injury were treated in the Rehabilitation Department of the Third Hospital of Hebei Medical University (Figure 1). The inclusion criteria were: age 16 to 65 years; strong awareness and compliance of rehabilitation; course of disease ranging from 0.5 to 12 months; no history of hip injury, bone and joint infection, and bone tumor; and no history of pelvis or femur fractures. Patients were excluded with the following diseases which may influence the level of α_2-HS glycoprotein: acute myocardial infarction (AMI), acute leukemia, chronic myeloid leukemia, myeloma cells and degeneration of bone marrow fibrosis, rheumatoid arthritis, lymphoma, alcohol hepatitis, liver cirrhosis, fatty liver, systemic lupus erythematosus, and Crohn's disease. The study was approved by the Ethics Committee of the Third Hospital of Hebei Medical University. Informed consent was obtained from all patients.

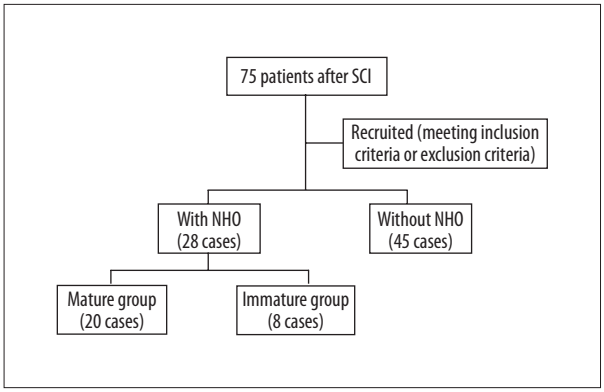

Figure 1. The flow chart of the retrospective study.

The mean age of the patients was 36.2 years, ranging from 16 to 58 years. The paraplegias were caused by traffic injury (18 patients), injury from a high fall (31 patients), crushing (11 patients), and other reasons (15 patients). The patients were divided into 2 groups according to the occurrence of heterotopic ossification based on the results of high-frequency ultrasound on the bilateral hip joint (Figure 2): Group A (patients with heterotopic ossification after spinal cord injury, 28 cases) and Group B (patients without heterotopic ossification after SCI, 47 cases).

Furthermore, the 28 cases in group A were divided into a mature group (20 cases) and an immature group (8 cases) according to the degree of maturity of neurogenic heterotopic ossification based on the results of high-frequency ultrasound (Figure 3). The ultrasonographic manifestations of mature NHO showed patchy hyperechoic lesion site, uneven surface, the rear sound shadow, boundary less clear, and unsmooth edge. Reactive thickening of the periosteum may be found adjacent to cortical bone, and sparse blood flow signals on color Doppler flow imaging (CDFI). The sonographic findings of immature NHO showed inhomogeneous and hypoechoic mass at the lesion, and the boundary was clear. The involved muscles can be localized, enlarged, and sometimes interrupted by muscle fibers. CDFI showed that the blood flow signals in the damaged muscle tissues increased more than that of the surrounding normal muscle tissues. With the extension of the disease, the signal of blood flow decreased gradually.

At the first visit, the sensory plane, the plane of motion, the muscle strength, the reflex, the joint activity, and the ADL (activities of daily living) ability were evaluated for all patients. We also instructed patients and their family members to do

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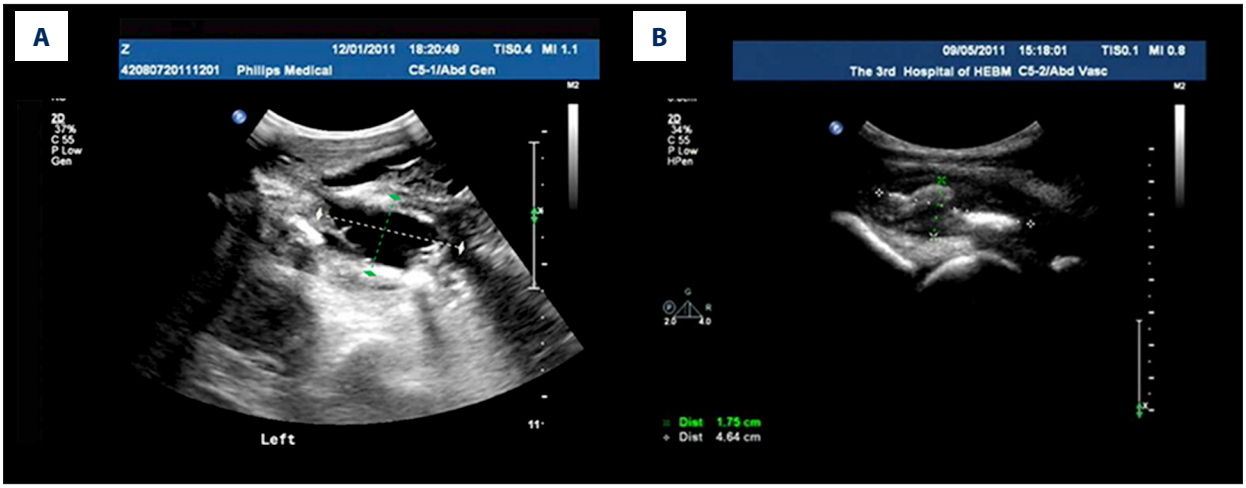

Figure 2. Representatives of sonographic findings of neurogenic heterotopic ossification. (**A**) Abnormal muscle structure: the anteromedial muscle group of left thigh showed structure derangement, and there was low and no echo area with irregular shape and island shape enhanced echo area (area indicated by dotted line in white and green). (**B**) Calcification: There were multiple strip or block enhanced echo areas (indicated by dotted line in white and green) with posterior acoustic shadowing in the anteromedial muscle group of left thigh.

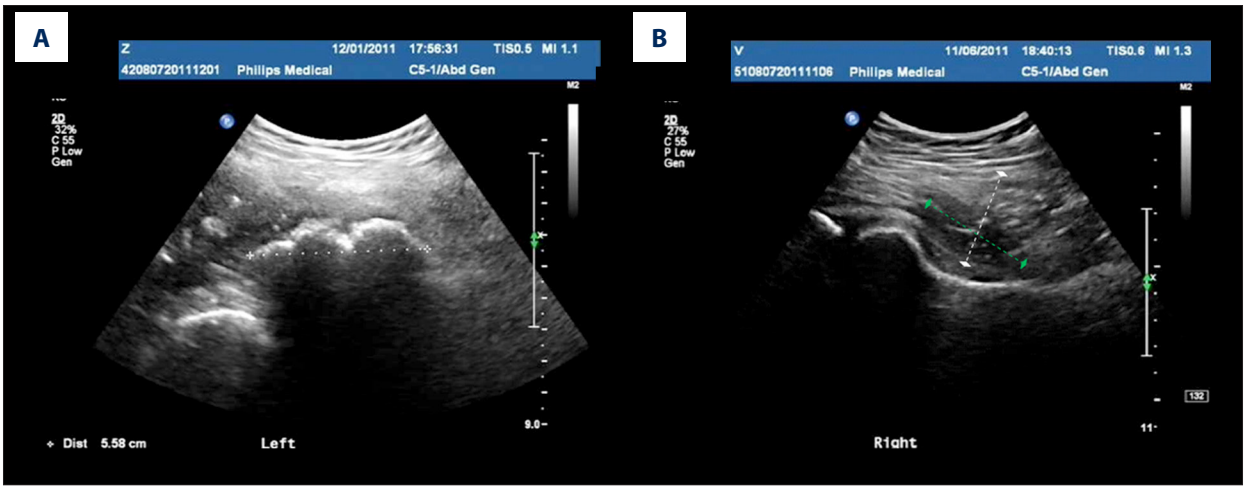

Figure 3. Representatives of sonographic findings related to the 2 stages of neurogenic heterotopic ossification. (**A**) Mature type: There were irregular lamellar and block enhanced echo areas with posterior acoustic shadowing in front of neck of femur in left thigh (dotted line in white). (**B**) Immature type: The thickened muscle and uneven low-level echo lump in front of neck of femur in left thigh (area indicated by dotted line in white and green).

physically active and passive activities in order to avoid contracture and joint mobility limitation due to joint disuse. The movements should be gentle to avoid joint soft tissue injury, hyperemia, hemorrhage, edema, and even organization which might induce or aggravate ectopic ossification.

Diagnosis of neurogenic heterotopic ossification

We used a Philips iU-22 ultrasound scanner with linear array probe with frequency of 5–10 MHz and convex array probe with frequency of 3.5–5 MHz for the diagnosis of neurogenic heterotopic ossification. The diagnosis of NHO used the ultrasonic zone phenomena proposed by Cassar-Pullicino [14]. Ultrasonography was carried out by the same doctor for all patients, and the diagnosis was done by 2 doctors. Dynamic imaging was stored and analyzed with QLAB software.

Blood specimens

Blood specimens (3 ml) were collected through the ulnar vein from all patients after overnight fasting for 10 h. After 30 min at room temperature, serum was separated by centrifugation at 3200 rpm for 6 min. The obtained serum specimens were then stored at 70°C for further use.

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Table 1. Demographics and clinical characteristics of patients.

Item	Group A (n=28)		Group B (n=47)		P value
Age (years)	45	(35)	34	(21)	>0.05
Male (n,%)	25	(89.3)	42	(89.3)	>0.05
Spasticity (n,%)	6	(21.4)	6	(12.8)	0.036
Pressure ulcer (n,%)	0		9	(19.1)	>0.05
Classification of ASIA (n,%)					0.033
A	28	(100)	40	(85.1)	
B	0		3	(6.38)	
C	0		4	(8.51)	
D	0		0		
Injury site (n,%)					>0.05
Cervical and upper thoracic injuries	9	(32.1)	17	(36.2)	
Middle and lower thoracic injuries	14	(50.0)	20	(42.6)	
Lumbar and below lumber injuries	5	(17.9)	10	(21.3)	
Location of NHO (n,%)					NA
Right hip joint	1	(3.57)	0		
Left hip joint	5	(17.9)	0		
Double hip joint	22	(78.6)	0		

NHO – neurogenic heterotopic ossification. The distribution of age was not normal. Therefore, the median (interquartile range) was used for description of the age for patients.

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was used to measure the level of α_2-HS glycoprotein, C-reactive protein (CRP), D-dimer, and bone morphogenetic protein (BMP), following the manufacturers' instructions. Thee ELISA kits were purchased from R&D Systems (USA).

Statistical analysis

Data were recorded and analyzed using SPSS 18.0 (Chicago, IL). Significant differences between the 2 groups were analyzed using the chi-square test or Wilcoxon ranking test, as appropriate. Multivariate regression analysis was used to analyze the relationship between serum α_2-HS glycoprotein and other factors.

Results

Demographics and clinical characteristics of patients

Group A (patients with heterotopic ossification after spinal cord injury) included 28 patients (25 male) with the mean age of 39.9 years. The position of heterotopic ossification was located at the right hip joint (1 patient), the left hip joint (5 patients), and double hip joints (22 patients). The mean time from the spinal cord injury to the observation of neurogenic heterotopic ossification was 2.73 months. According to the paraplegia plane and spinal cord injury site, there were 9 cases with cervical and upper thoracic injuries, 14 cases with middle and lower thoracic injuries, and 5 cases with lumbar and below lumber injuries. The paraplegia plane was consistent with injury level in 22 cases, and the paraplegia plane was higher than injury level in 6 cases.

Group B (patients without heterotopic ossification after SCI) included 47 patients (42 male) with the mean age of 33.9 years. According to the paraplegia plane and spinal cord injury site, there were 17 cases with cervical and upper thoracic injuries, 20 cases with middle and lower thoracic injuries, and 10 cases with lumbar and below lumber injuries. The paraplegia plane was consistent with injury level in 45 cases, and the paraplegia plane was higher than injury level in 2 cases.

The demographics and clinical characteristics of patients are given in Table 1, showing there was no significant difference in age and sex between the 2 groups (p>0.05). The proportion of patients with pressure ulcers was significantly higher in SCI

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Table 2. The serum parameters summary in group A and group B.

Parameter	Group A	Group B	Z value	P value
Ca2+	41.55 (65.61)	10.32 (3.39)	–5.17	<0.001
D-dimer	83.54 (142.54)	24.18 (12.81)	–5.362	<0.001
AHSG	47.47 (13.13)	54.48 (19.18)	–2.169	0.03
CRP	1611.81 (2063.51)	317.30 (218.69)	–5.094	<0.001
BMP	8.49 (12.91)	2.11 (0.47)	–5.204	<0.001

Table 3. The serum parameters summary in mature group and immature group.

Parameter	Group A	Group B	Z value	P value
Ca2+	45.79 (93.14)	37.37 (62.51)	0.000	0.999
D-dimer	98.46 (174.30)	78.96 (101.88)	0.000	0.999
AHSG	47.38 (12.28)	54.01 (24.45)	–0.840	0.401
CRP	1664.65 (2678.25)	1611.81 (1771.84)	–0.967	0.334
BMP	8.46 (16.52)	8.68 (11.63)	–1.017	0.309

Table 4. The correlation between α_2-HS glycoprotein and other factors (multiple linear regression).

Influential factor	Regression coefficient	Standard error	Standardization regression coefficient	t value	P value
Constant item	49.794	10.756		4.629	0
CRP	0.015	0.007	0.253	2.265	0.027
Spasticity	34.411	17.089	0.225	2.014	0.048

patients with NHO than in SCI patients without NHO (p=0.036). No significant difference was found in the proportion of patients with spasticity (p>0.05). There was a very significant difference of injury degree between the 2 groups. According to the ASIA impairment scale classification, all patients in group A were classified into ASIA grade A, while only 85.1% of the patients in group B were ASIA grade A. There was no significant difference between group A and group B in injury site (p>0.05).

Decrease of α2-HS glycoprotein in SCI patients with neurogenic heterotopic ossification

Our results showed that the levels of α_2-HS glycoprotein, CRP, serum calcium, D-dimer, and BMP in SCI patients with NHO were all significantly different from those in SCI patients without NHO (Table 2). A closer look at the data revealed a significantly lower level of α_2-HS glycoprotein (p=0.03) in SCI patients with NHO as compared with SCI patients without NHO. In contrast, compared to SCI patients without NHO, a significant elevation of serum calcium (p<0.001), D-dimer (p<0.001), BMP (p<0.001), and CRP (p<0.001) was observed in SCI patients with NHO (Table 2).

Degree of maturity of NHO did not influence the level of α_2-HS glycoprotein

We investigated also whether there was a difference in levels of α_2-HS glycoprotein, CRP, serum calcium, D-dimer, and BMP in different degrees of maturity of neurogenic heterotopic ossification. The levels of α_2-HS glycoprotein, CRP, serum calcium, D-dimer, and BMP in the mature group and immature group of group A were compared using the Wilcoxon ranking test. The result indicated that there was no significant difference in α_2-HS glycoprotein, CRP, serum calcium, d-dimer, and BMP between the 2 groups, indicating that there was no relationship between the level of α_2-HS glycoprotein, CRP, serum calcium, d-dimer, and BMP and the degree of maturity of neurogenic heterotopic ossification (Table 3).

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Multivariate liner regression analysis

According to the analysis of serum α_2-HS glycoprotein level and other factors by multiple linear stepwise regression analyses, there was no correlation between serum α_2-HS glycoprotein level and age, pressure sores, BMP, D-dimer, and serum calcium, but we found a positive correlation between serum α_2-HS glycoprotein level and CRP and spasticity (Table 4).

Discussion

We studied a group of 75 patients with paraplegia caused by spinal cord injury, with or without the presence of NHO. We found that the levels of α2-HS glycoprotein, CRP, serum calcium, d-dimer, and BMP in SCI patients with NHO were all significantly different from those in SCI patients without NHO. A significant decrease of α2-HS glycoprotein in SCI patients with NHO was found, as compared with SCI patients without NHO. The degree of maturity of NHO did not influence the level of α2-HS glycoprotein. Multivariate liner regression analysis showed that the level of serum α2-HS glycoprotein was correlated with CRP and spasticity. Our findings suggest that the decreased level of α2-HS glycoprotein may be related to the formation of neurogenic heterotopic ossification in patients with spinal cord injury, and a decreased level of α2-HS glycoprotein might be a risk factor for NHO in patients with SCI.

After spinal cord injury, the humoral factors, nerve immune function, metabolism function, and the local microenvironment all change, but the exact mechanism by which these factors induce the formation of NHO is still not clear. Chalmers et al. proposed that the formation of heterotopic ossification must have the following 3 conditions: osteogenic induction, osteogenic precursor cells, and an environment which allow the osteogenesis [15]. According to Kaplan et al., 4 elements are required for heterotopic ossification: 1) local edema around the joint after SCI (initial events); 2) local infiltration of inflammatory cells and release of cytokines (efferent signal); 3) migration of mesenchymal stem cells, accumulation of osteoblasts, production of tropocollagen which aggregated into collagen, and new bone formation (stem cells with osteogenic potential); and 4) local abnormal changes in nutritional status after SCI, local circulation dynamic changes in the tissues, decreased PO2, microvascular dysfunction, and independent nerve dysfunction, providing a suitable environment for the growth of ectopic bone tissue organization (a suitable environment). Among the above elements, the signal factors are most important for elaborating the metabolic effects on NHO after SCI. This was why we chose the regulatory protein α_2-HS glycoprotein to study the effect of heterotopic ossification after spinal cord injury.

The α_2-HS glycoprotein has been considered to be the major calcification inhibitor in the extracellular space [9,11,12]. Jahnen-Dechent et al. found a significant inhibition of calcification in mice knocked-out of α_2-HS glycoprotein, and severe micro-calcification was found in soft tissue and blood vessels [12]. *In vitro* experiments have also confirmed that the α_2-HS glycoprotein dose-dependently inhibits calcification of bovine vascular smooth muscle [16,17]. Although there are many reports in this field, less is known about the effect of alpha 2-HS glycoprotein on heterotopic ossification, especially in SCI patients with NHO. In our study, we found that the serum level of α_2-HS glycoprotein was significantly lower in SCI patients with NHO as compared with SCI patients without NHO. This indicates that the decrease of serum level of α_2-HS glycoprotein may be related to the formation of NHO. It should be noted that we found no significant difference in different degrees of maturity of neurogenic heterotopic ossification, which suggests that the level of α_2-HS glycoprotein may be not associated with the maturity degree of neurogenic heterotopic ossification. However, due to the limited size of our sample, further studies with larger sample sizes are needed to verify these results.

Wittenberg et al. [18] reported that the development of NHO is related to the site of injury, regardless of the severity and occurrence of SCI, which means that the incidence rate NHO in cervical and thoracic spinal cord injury patients is higher as compared with other types of injury with low paraplegia plane. However, NHO mostly occurs in severe SCI patients (ASIA A neurological classification and ASIA B neurological classification). In patients with only loss of sensory function, none developed NHO in our group of cases. Our results suggest the formation of NHO is significantly correlated with the severity of SCI, but is not associated with the location of SCI. Bravo-Payno et al. [6] reported that the incidence rate of NHO in patients with spasm was significantly higher than in those without spasm. However, we found that the formation of NHO and degree of maturity of NHO were not correlated with increased muscle tension (spasm) after injury. This result may be caused by the relatively small sample size in our study, and further studies enrolling more patients are needed.

Deep venous thrombosis (DVT) is a common complication of SCI, with clinical features of lower-limb swelling and edema. Serum D-dimer is a breakdown product of fibrin, which serves as a useful diagnostic marker for DVT [19–22]. It has been reported that the incidence rate of DVT was 6.55% in acute SCI patients, and the incidence rate of NHO was 8%, with a correlation between the DVT and NHO [23]. Perkash et al. emphasized that NHO can cause a secondary hypercoagulation state in patients with spinal cord injury, and thus lead to the occurrence of DVT [24]. In agreement with these findings, in our study, 11 of the 28 (39.3%) SCI patients with NHO had

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DVT, and the level of D-dimer was significantly higher these patients than in SCI patients without NHO.

C-reactive protein (CRP), a nonspecific biomarker of inflammation, is upregulated in acute trauma or infection [25]. It is a positive acute-phase reactant. Unlike CRP, α_2-HS glycoprotein is a negative reactant. Both variables represent the same biological event, and thus are considered to be inflammation-related variables [26]. It has been reported that α_2-HS glycoprotein is an alternative to CRP in the Würzburg dialysis cohort, and is inversely correlated with CRP concentrations [26]. Our results showed that the level of CRP was positively correlated with the occurrence of NHO after spinal cord injury, which accords with published data [27].

Conclusions

In conclusion, we found that decreased level of α_2-HS glycoprotein may be related to the formation of neurogenic heterotopic ossification in patients with spinal cord injury, and the level of α_2-HS glycoprotein was not influenced by the degree of maturity of NHO. Our results suggest that decreased levels of α2-HS glycoprotein might be a risk factor for NHO in patients with SCI, which may have potential benefits for patients with SCI though early monitoring and diagnosis of NHO. However, the sample size in this study is relatively small. Further well-designed clinical studies with larger sample sizes and long-term follow-up are needed to elucidate the actual role of α_2-HS glycoprotein in NHO.

Conflict of interest

None.

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