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PhD THESIS

THE EFFECTS OF SMOKELESS TOBACCO ADMINISTRATION ON PERCEPTION OF EFFORT AND COGNITIVE TASK IN MEN

S.S.D BIO14/FARMACOLOGIA

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Dedicated to Giovanni Zandonai
named “Gianetto”, a free man

CONTENTS

Abstract

Some evidence suggests that smokeless tobacco (snus) is widespread among athletes from countries where a general population use it. A proliferation of nicotine use in the sport environment has been observed in recent years. Thus, nicotine has been placed on World Anti-doping Agency's (WADA) 2013 Monitoring Program.

In literature it is little known regarding the actual effects of this product, especially on endurance performance, on a cognitive performance and on football players' performance. Therefore, the main objective of the present thesis was to study the effects of snus administration during these three performances. To achieve this goal we designed four studies.

First of all, we performed two surveys (STUDY I) to investigate snus past experience and current use (Survey I) and reinforcement effects of snus (Survey II) in a sample of winter sport athletes in Northern of Italy. Data collection was performed by administering a questionnaire to winter sport athletes after competitive ski races or during training in Italian Dolomites (Northern Italy). Smoking status and dual snus-smoking use information were also collected. Eighty out of a one hundred-eight participants (74%; 80.5% of males and 61.1% of females) tried snus at least once. Fifty-four participants were current snus users (50% out of the total). Forty-one participants were current smokers (38%). Smoking status and reinforcement effects of snus use (Survey II) information were also collected with the modified Cigarette Evaluation Questionnaire (mCEQ). Comparison between occasional vs. regular snus users (61 subjects) showed a statistically significant difference for satisfying ($p<0.0088$), calm ($p<0.0252$) and enjoying ($p< 0.0001$) mCEQ items. Survey I data confirmed snus use among athletes, and showed a higher smoking prevalence in this sample than in the general population. Survey II confirmed that snus use is reinforcing in current snus users. The findings were collected among athletes coming from a country where there is not socio-cultural snus tradition and where marketing is banned. In this STUDY I we hypothesize that both a successful 'sport' role–model, and the availability of accepted and flexible nicotine 'multi-delivery' may be the determinant factors of this phenomenon.

The aim of the second study (STUDY II) was to firstly investigate the effects of snus on the perception of fatigue during an endurance exercise in men. The study was a double-blind placebo controlled crossover design study. We recruited 14 male non-

smokers and non-snus users. Subjects were studied during three sessions on cycle-ergometer: experiment 1 (EXP1) consisted on an incremental exercise test to determine Wmax (maximal aerobic power output); EXP2 and EXP3 consisted on snus or placebo administration followed by an exercise at 65% Wmax until exhaustion. During the EXP2 and EXP3 the global rating of perceived exertion (RPE) was recorded, using the 15-point Borg scale. Before and after all experiments, subjects were administered the Profile of Mood of State questionnaire (POMS) and tested by means of Transcranial Magnetic Stimulation (TMS) to assess changes in cortico-motor excitability due to the prolonged exercise. In this STUDY II we observed that snus does not change RPE compared to placebo condition; this means that the sought effect could not be an improvement of fatigue during an endurance exercise until exhaustion.

In the third study (STUDY III) our aim was to measure the effect of snus administration on the Iowa Gambling Task (IGT) an experimental test to study decision-making process. We recruited 40 male non-smokers and non-snus users. Subjects were randomized to blindly receive snus or placebo on two different days according to a cross-over design. No significant differences were observed during the IGT performance under both conditions (snus vs. placebo).

In the last study (STUDY IV) we assessed the effect of Snus on physical performance, heart rate variability, subjective arousal, and mental workload in non-smokers non-snus user amateur football players. Participants were administered either snus or placebo forty minutes prior to a fitness test battery. Hearth rate values, global ratings of perceived exertion, perceived arousal and, perceived mental workload were collected after the snus or placebo administration. The fitness test battery consisted of 4 tests: Handgrip Test, Counter-movement Jump, Agility test and Yo-yo intermittent recovery test. Significant differences were observed in agility test performance (18.82 ± 0.81 vs. 18.47 ± 0.62 seconds), level of mental fatigue before the experimental session (4.17 ± 2.38 vs. 2.94 ± 1.89 points), and perceived mental load after the overall experimental session (6.37 ± 2.16 vs. 5.44 ± 1.83 points) (snus vs. placebo conditions, respectively). The outcome of the STUDY IV suggested that snus, due to its detrimental effects on performance, is counter-indicated as an ergogenic aid.

This research project aimed to assessed the effects of snus administration in non-smokers non-snus users healthy male subjects on endurance performance, cognitive performance and football players' performance showed that snus administration (vs. placebo condition) did not improve exercise and cognitive performance.

List of studies

The present thesis is based on the following studies, which are referred to in the text by their Roman numerals:

STUDY I Smokeless tobacco (snus) use among winter sport athletes in Italy

STUDY II Effects of snus administration on exercise endurance in males *

STUDY III Effects of snus administration in Iowa Gambling Task in males **

STUDY IV Acute effect of snus on physical performance and perceived cognitive load on amateur male footballers ***

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List of abbreviations

AC	Abstinence Condition
AT	Agility test
BMI	Body Max Index
CI	Confident Interval
CMJ	Countermovement jump
CO	Carbon Monoxide
CO2	Carbon dioxide
CQ	Control Questions
DM	Decision-Making
ESI	Positive Electrospray Ionisation
EU	European Union
EXP1	Experiment one
EXP2	Experiment two
EXP3	Experiment three
F.I.S.I.	Italian Winter Sports Federation
FTB	Fitness Test Battery
HDT	Hand dynamometric test
HHb	Deoxy-haemoglobin
HPLC	High Performance Liquid Chromatography
HR	Heart Rate
HRV	Heart Rate Variability
IGT	Iowa Gambling Task
Mb	Myoglobin
mCEQ	Cigarette Evaluation Questionnaire
MD	Medical Visit
N	Number of participants
NIRS	Near-infrared Spectroscopy
O2Hb	Oxy-haemoglobin
OST	Oral Smokeless Tobacco
POMS	Profile of Mood of State questionnaire
PR	Perceived Readiness
Q’	Cardiac output
RER	Respiratory Exchange Ratio

rMSSD	Root-mean-square difference of successive normal R-R intervals
RPE	Rating of Perceived Exertion
RPM	Revolutions per minute
Rri	R-R interval
SC	Satiety Condition
SD	Standard Deviation
SE	Standard Error
SEI	Side Effects interview
SIM	Selected Ion Monitoring
SO	Substance (Snus/Placebo) Out
SP	Swedish Snus Placebo
SS	Swedish Snus
STPs	Smokeless Tobacco Products
SV	Stroke Volume
T0	Time zero
T1	Time one
THb	Total haemoglobin
Tmax	Maximum Time
TMS	Transcranial Magnetic Stimulation
$\dot{V}CO_2$	Rate of CO_2 production
$\dot{V}E\ O_2$	Expiratory minute ventilation
$\dot{V}O_2$	Consumption Rate
VAS	Visual Analogue Scale
VL	Vastus lateralis muscle
VO_{2max}	Maximal Oxygen Uptake
W	Watt
WADA	World Anti-Doping Agency
Wmax	Maximal aerobic power output
YYT	Yo-yo recovery intermittent test
[G]b	Blood glucose concentration
[Hb]b	Blood haemoglobin concentration
[La]b	Blood lactate concentration

1. GENERAL INTRODUCTION

Smokeless tobacco (ST) is taken orally or nasally without burning the product at the time of use. Oral smokeless tobacco (OST) products are placed in the mouth, cheek or lip and sucked ('dipped') or chewed. Nicotine is considered to be a major addictive component in smokeless tobacco products (STPs) and the total nicotine content in different STPs varies, depending on various factors, including the kind of tobacco used (Ramström 2000). Nicotine is a psychomotor stimulant and this raises the issue of whether such use constitutes that of a performance-enhancing drug (Chiamulera et al., 2007). One way of consuming nicotine is through the use of snus.

1.1 Snus

Smokeless Tobacco Commission (ESTOC) has defined snus as, "*an oral smokeless tobacco product traditionally used in Sweden that is manufactured using a tobacco heat- treatment process*" (Rutqvist et al., 2011). Snus is an air-cured, finely ground, heat pasteurized tobacco product and it is marketed in portion-bag packets (pouches) (Figure 1), in a variety of flavors (Andersson et al., 1995; Lunell and Lunell 2005). This OST has always been the predominant form of ST in Sweden, which is the only country in Europe that can sell legally snus (Anon 1992).

Figure 1. Portion-bag packets of snus.

The snus is typically held in the mouth between the upper lip and the gum for approximately 30-45 minutes before it is thrown out (Foulds et al., 2003). The dose of nicotine cannot be predicted simply from the nicotine content of cigarettes, tobacco, or smokeless tobacco products (Benowitz and Jacob 1984). Nicotine is absorbed rapidly when tobacco smoke reaches the small airways and alveoli of the lung. It takes 10-19 seconds for nicotine to pass through the blood-brain barrier (Benowitz 1999). In contrast, the use of smokeless tobacco products results in a gradual rise of nicotine blood concentrations, with little arterial-venous disequilibrium (Le Houezec and Benowitz 1991). Nicotine blood levels through smokeless tobacco paths of nicotine intake tend to reach a peak after about 30 minutes, with the levels continuing and decreasing slowly over 2 or more hours (Benowitz 1999; Hukkanen et al., 2005). Foulds et al (2003) showed that nicotine plasma levels from smoking the cigarette rose more rapidly than for the oral products (Figure 2). According to Lunell and Curvall (2011) the mean time to maximum nicotine plasma concentration (*T*max) is 37.1 ± 10.2 min (range: 24–60 min) for 1 g portion (8.7 mg nicotine) of snus.

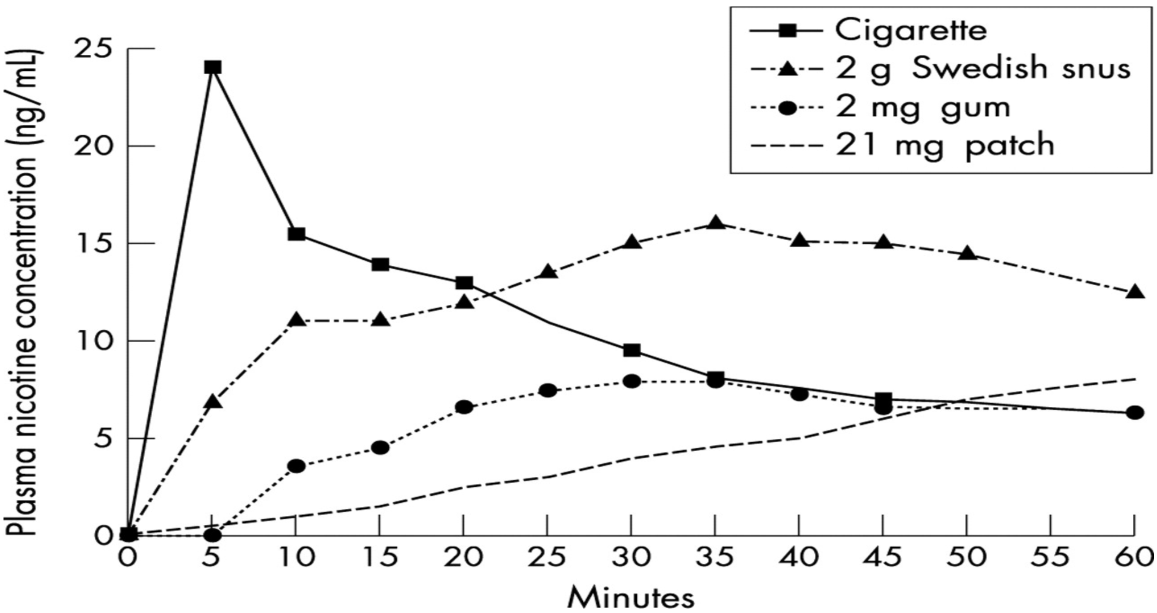

Figure 2. Nicotine plasma concentration in cigarette and STPs. Venous blood concentrations in nanograms of nicotine per milliliter (ng/ml) of plasma as a function of time for various nicotine delivery systems. Image taken from Foulds et al 2003.

Andersson et al (1994) showed that 10-20 % of the nicotine originally present in the snus is absorbed via the buccal mucosa and reaches the systemic circulation (Andersson et al., 1994; Andersson et al., 1995). Therefore, only 1-2 mg of nicotine is absorbed into the blood from a one-gram pinch containing approx. 10 mg of nicotine. The amount of nicotine absorbed varies greatly among individuals. Lunell and Lunell (2005) observed that the variation in extraction rates among different snus users was 50 to 300% greater than the variation from portion to portion in the same snus user. The percentage of nicotine extracted from snus is 22%-44% (Digard et al., 2013; Lunell and Lunell 2005). The prolonged elimination of nicotine in snus users has been attributed to continued absorption of nicotine released from the mucous membrane or to absorption of nicotine that has been swallowed (Figure 3) (Benowitz et al., 1989; Lunell and Lunell 2005).

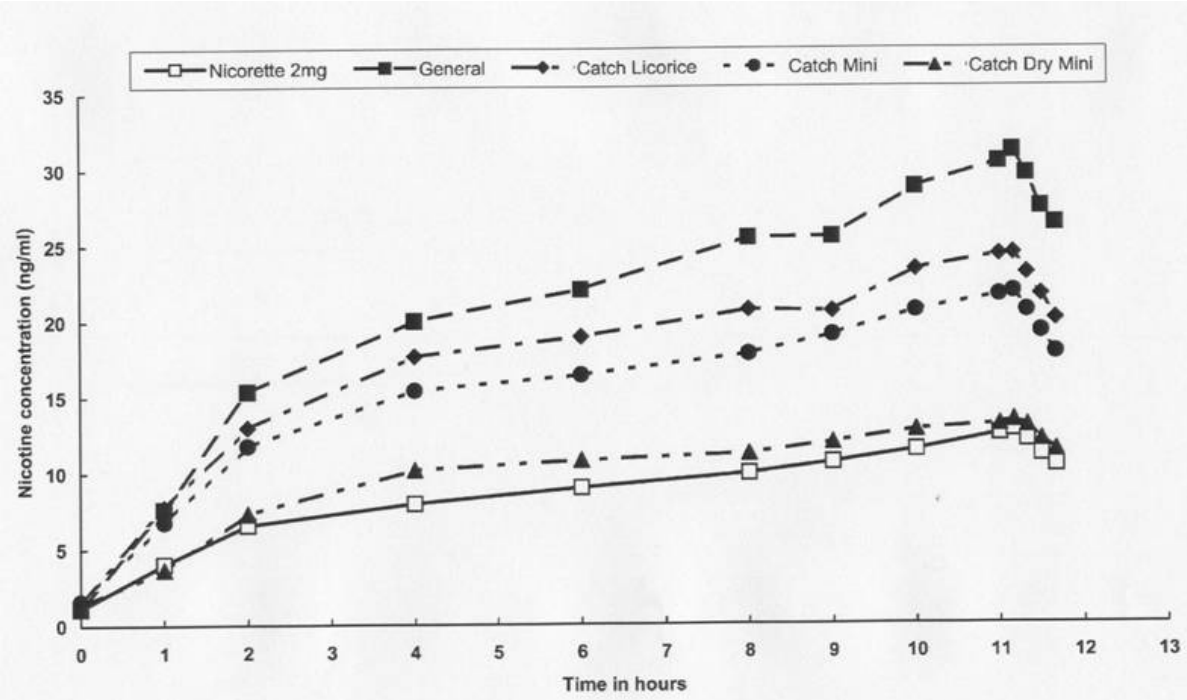

Figure 3. Nicotine plasma concentration–time curves of STPs. Mean nicotine plasma concentration–time curves obtained following hourly use of four different brands of Swedish snus and 2-mg Nicorette chewing gum. Image taken from Lunell and Lunell 2005.

The use of snus among young people has been increasing in countries such as Norway (SSB 2013), Sweden (Norbert 2013) and in the USA (Bhattacharyya N 2012). In literature are presents some studies about differences in gender for snus use (Digard et al., 2009; Huhtala et al., 2006, Wiium et al., 2009), with a lower number of female snus users, and most of them self-reporting less attractiveness of snus compared to other forms of tobacco use. It is interesting to note that in these surveys the prevalence of snus use among females was greater than that reported in the general female population of other countries (e.g., Sweden see Digard et al.,

2009) even if the small sample size cautions about a comparison.
Some studies showed a high use of a psychoactive substance (nicotine) through snus, among athletes in a winter sporting environment (Zandonai et al., 2013; Marclay and Saugy 2010: Marclay et al., 2011). Three studies showed high levels of snus use among Scandinavian athletes (Mattila et al., 2012; Alaranta et al., 2006; Martinsen et al., 2012), and in U.S sport environment (Green et al., 2001; Severson et al., 2005). Chiamulera et al (2007) argued whether "*subjects who are taking ST benefiting from legal doping?*". He suggested that ST effects should be tested on field parameters, which are relevant to the specific desired effect in sports rather than the 'traditional' psychometric measures. But only from 2012, nicotine has been placed on WADA's 2012 Monitoring Program in order to detect potential patterns of abuse.

In literature it is observed a snus use in sports environment. In contrast to this there is not literature that show the effect of snus on sport and cognitive performance. There are many articles about nicotine effects administered with OST as nicotine gum (Hindmarch et al., 1990; Sherwood et al., 1992; Parrot and Winder 1989; Philips and Fox 1998). Other hand, findings on the effects of nicotine in healthy abstinent smokers and non-smokers have been inconsistent. While some studies have shown a reduction in reaction time and an increase in accuracy scores following smoking or administration of 2 mg of nicotine via other routes in acutely abstinent smokers, satiated smokers and non-smokers (Kerr et al., 1991; Sherwood et al., 1992; Houlihan et al., 2001), others have shown no effect of subcutaneous injections of 2 or 4 mg nicotine gum on this task in similar groups (Hindmarch et al., 1990; Foulds et al., 1996). Another group has reported impaired accuracy in abstinent smokers immediately following smoking (Spilich et al., 1992). In a another study using this task by Grobe et al (1998), half of the memory sets were presented concurrently with a distracting tone. Nicotine nasal spray, in comparison to placebo, significantly reduced scanning rates in the presence of distracting stimuli, in acutely abstinent smokers but not in non-smokers. In addition, while Foulds et al (1996) found no effect of subcutaneous nicotine on digit recall in abstinent smokers, non-smokers recalled more digits after experimental administration of nicotine than saline. Finally, neither Powell et al (2002) nor Jones et al (1992) found an effect of smoking or subcutaneous nicotine injection on reverse or forward digit span in either acutely abstinent smokers or non-smokers.

1.2 Aim of the thesis

Most of sport practice has developed empirically and sports insiders have not yet realized the importance to appraise the evidence base, especially when using substances. We have also to emphasize that in literature there are few studies that have shown substances effectiveness (e.g. cognitive enhancer administration). In fact, there are anecdotal, but sparsely experimental, evidence for efficacy in healthy subjects: people taking cognitive enhancer drugs subjectively report a perceived improvement of performance. In spite of lack of objective evidence, the use is increasingly widespread (Maher 2008; Chiamulera 2011).

Considering these considerations and the gaps in literature, the current body of work was designed to investigate four aspects of snus administration in healthy male participants. Therefore, four main issues were addressed:

1. How many athletes use snus in a country with no socio-cultural tradition and marketing of snus?
2. Does snus administration increase endurance performance and perception on effort?
3. Does snus administration change Decision-Making process?
4. Does snus use affect physical performance, heart rate variability, and perceptual responses in amateur footballers?

To answer these questions, we performed four studies.

In the first study (STUDY I) we explored snus use by investigating self-report past snus experience and current use in a sample of winter sport athletes in Northern Italy. In the same study we aimed to know the pattern of snus-induced reinforcing effects subjectively described by skiers.

In the STUDY II we investigated the snus effects on exercise endurance correlated with the perception of fatigue along with a range of physiological and neuro-physiological parameters.

The STUDY III investigated the effect of snus administration in IGT as a validated tool to study Decision-Making process.

Finally, in the STUDY IV we assessed the effect of snus on physical performance, heart rate variability, and subjective arousal and mental workload in non-smokers non-suns users amateur football players.

2. STUDY I

Smokeless tobacco (snus) use among winter sport athletes in Italy

2.1 Introduction

Smokeless tobacco is a form of tobacco use that is widespread in North America and Scandinavian countries such as Sweden, Norway and Finland. In particular, in the latter countries, the moist snuff form called 'snus' is the most widely used and popular among young people (Huhtala et al., 2006; Stenbeck et al., 2009).

The prevalence and the socio-demographic patterns of snus use have been extensively investigated and described (Digard et al., 2009; Furberg et al., 2006; Lundvqvist et al., 2009; Overland et al., 2010). In Italy, although snus is not marketed in accordance to the European Union ban, (Anon, 1992; Ahlbom et al., 2007) it could be however purchased via Internet. Recently, there were some anecdotal reports about the use of snus in alpine skiers participating at ski championship races across the alpine area, Europe. A preliminary survey, which we conducted during competitive events in the Italian Dolomites, showed that approximately half of the interviewed skiers were current snus users. The use of snus in this particular segment of young population is somehow surprising considering that it takes place in a country where there is not socio-cultural tradition and marketing of snus.

Moreover, there are currently unanswered psychobiological questions such as the sought and the subjective reinforcing effects experienced by this specific athletic population. In fact, from a psychobiological standpoint, snus is a delivery of a psychoactive and addictive substance, nicotine (Fagerstrom and Schildt, 2003; Henningfield et al., 1997). Snus users have positive expectancies about effects (Wiium and Aaro 2011), report subjective pleasure (Caldwell et al., 2010), exhibit dependent behavior and withdrawal symptoms (Hatsukami et al., 1987; Timberlake 2008). Considering the existence of dual snus-smoking users, a 'multi-delivery', flexible, pattern of nicotine self-administration is the psychobiological mechanism that could explain snus use behavior (Post et al., 2010).

The general aim of our investigation is to further extend our preliminary observations about the snus use phenomenon, and to investigate the potential psychobiological reasons: why this segment of Italian winter sport athletes is taking snus? Therefore, the primary scope of Survey I was to explore this phenomenon by investigating self-report past snus experience and current use in a sample of winter sport athletes. The Survey II aimed to know the pattern of snus-induced reinforcing effects subjectively described by skiers. Considering the lack of background knowledge about snus use in Italy, these were pilot surveys, which were designed without any ad-hoc assumption about social models, gateway hypothesis or other inductive questions.

2.2 Materials and methods

2.2.1 Participants

In Survey I we interviewed 108 athletes who practice winter sports. One-hundred-five participants were agonistic athletes (97.2%), i.e. being in possession of the medical certificate of fitness in order to participate in agonistic races organized by Italian Federation of Winter Sports (F.I.S.I.). The three winter sports were alpine skiing, nordic skiing and biathlon (i.e., rifle shooting and nordic skiing).

2.2.2 Instruments

The Survey I questionnaire was divided into three main sections. First section investigated demographic data, the winter sport(s) practiced, age of start, agonistic level, and other practiced sports. Section two collected self-report data about snus use. Past snus experience was asked with the questions: “Have you ever tried snus? How many times in your lifetime?’’ The answers were 1 ‘‘I have never tried’’, 2 ‘‘I have tried once’’, 3 ‘‘I have used snus 2–50 times’’, or 4 ‘‘I have used snus more than 50 times’’. Answers 2 or 3 were defined as ‘limited snus experience’, whereas answering to “…more than 50 times” (Answer 4) was defined as ‘extensive snus experience’. The age of the first snus experience was also recorded. The current snus use was asked by the question ‘‘Do you use snus at present?’’ with answers ‘‘not at all’’, ‘‘occasionally’’ (less than once a day, defined as ‘occasional use’), or, ‘‘once a day or more often’’ (defined as ‘regular use’) (Huhtala et al., 2006). Section three investigated current smoking status. At the question “Do you smoke at the present?”, answers were “no”, “occasionally” (less than one cigarette a day, defined as ‘occasional smoking) and “yes” (one or more cigarette a day, defined as ‘regular smoking’). The number of cigarettes smoked per day was also recorded.

We collected data after two competitive races of alpine skiing (80% of the participants) or during training (20% of the participants) in Italian Dolomites (Northern Italy). Each questionnaire was made anonymous by an alphanumeric code (2-letter name initials and 8-digit date of birth). Gender differences in prevalence were analyzed by contingency table analysis with Fisher's exact test (GraphPad Prism 4). Analysis was reported as odds ratios and confidence intervals (C.I.).

In Survey II sixty-one participants who reported past or current snus use were included. Questionnaire was divided into three main sections. First section investigated demographic data, the winter sport(s) practiced, the age of start, the agonistic level and any other practiced sports.

Section two collected self-report data about age of the first snus experience. The current snus use was asked by the question ''Use snus now?'' with answers ''no'', ''occasionally'' (less than once a day, defined as 'occasional use'), or, ''once or more a day'' (defined as 'regular use') [1]. Reinforcing effects of snus was investigated with the items: 1 "Was using snus satisfying?", 2 "Did snus taste good?", 3 "Did you enjoy the sensations in your throat and chest?", 4 "Did using snus calm you down?", 5 "Did using snus make you feel more awake?", 6 "Did using snus make you feel less irritable?", 7 "Did using snus help you concentrate?", 8 "Did using snus reduce your hunger for food?", 9 "Did using snus make you dizzy?", 10 "Did using snus make you nauseous?", 11 "Did using snus immediately relieve your craving for a cigarette?", 12 "Did you enjoy using snus?". These items are rated on a seven-point scale ranging from 1 (not at all) to 7 (extremely) (Caldwell et al., 2010; Cappelleri et al., 2007). Section three investigated current smoking status. At the question "Do you smoke at the present?", answers were "no", "occasionally" (less than one cigarette a day, defined as 'occasional smoking) and "yes" (one or more cigarette a day, defined as 'regular smoking').

The number of cigarettes smoked per day was also recorded. The questionnaire used to collected data was the modified Cigarette Evaluation Questionnaire (mCEQ) [13] with 12 items: 1 "Was smoking satisfying?", 2 "Did cigarettes taste good?", 3 "Did you enjoy the sensations in your throat and chest?", 4 "Did smoking calm you down?", 5 "Did smoking make you feel more awake?", 6 "Did smoking make you feel less irritable?", 7 "Did smoking help you concentrate?", 8 "Did smoking reduce your hunger for food?", 9 "Did smoking make you dizzy?", 10 "Did smoking make you nauseous?", 11 "Did smoking immediately relieve your craving for a cigarette?", 12 "Did you enjoy smoking?". These items are rated on a seven-point scale ranging from 1 (not at all) to 7 (extremely).

According to Cappelleri et al., (2007) the twelve items are grouped in five domains: Satisfaction with three items (items 1, 2, and 12); Psychological Reward with five items (items 4 through 8); Aversion with two items (items 9 and 10); Enjoyment of Respiratory Tract Sensations (Item 3); and Craving Reduction (item 11). Scores for each subscale were calculated as the average of its individual item responses. Higher scores indicated greater intensity of each snus/smoking effect.

Questionnaires were administered after competitive races or during training in Italian Dolomites (Northern Italy) and were made anonymous by an alphanumeric code (2-letter name initials and 8-digit date of birth).

In according with Italian law, in the case of administration of questionnaires to healthy voluntary participants, does not require the approval of the ethics committee.

Data were analyzed by Mann Whitney test and unpaired t test analysis (GraphPad Prism 4).

2.3 Results

In Survey I among 108 participants, there were more males than females (72 vs. 36) (Table 1). The mean age was the same for both gender subgroups, with an overall average value of 23.9 years (± 2.6 SD). Except for 3 participants, all were agonistic winter sport athletes (97.2%), with all of them starting activity at a very young age (5.7 ± 2.5, years of age ± SD). Ninety-two participants (85.2%) did more than one exercise or sport activity (e.g., cycling, running) other than the main winter sport (Table 1).

Eighty athletes of the total (74%; 80.5% of males and 61.1% of females) tried snus at least once. Contingency table analysis shows a significant gender difference, with a higher risk to experience snus if male (odds ratio = 2.636 [C.I. 1.084, 6.412]; $p = 0.037$). Fifty-nine athletes had a limited past experience (ranging from 1 to 50 times) whereas 21 took snus more than 50 times. The first snus experience took place at an average age of 17.3 years (± 1.9 SD), with no significant difference between males and females (Table 1).

Table 1. *Survey I. STUDY I. Demographics of participant and prevalence of past snus experience.*

	Totals [%]	Male [%]	Female [%]
Participants, *N*	108 [100]	72 [67.0][a]	36 [33.0][a]
Age in years, mean (± SD)	23.9 (±2.6)	24.3 (±2.8)	23.3 (±1.8)
Athletes performing agonistic winter sports, *N*	105 [97.2]	69 [95.8]	36 [100]
alpine skiing	83 [76.8]	53 [73.6]	30 [83.3]
nordic skiing	23 [21.3]	17 [23.6]	6 [16.7]
biathlon	2 [1.9]	2 [2.8]	0 [0.0]
Age of starting winter sport, mean years (± SD)	5.7 (±2.5)	5.9 (±2.8)	5.4 (±2.0)
Athletes performing supplementary exercise or sport activity, *N*	92 [85.2]	65 [90.3]	27 [75.0]
Past snus experience, *N*	80 [74.0]	58 [80.5]	22 [61.1]
limited (1 to 50 times)	59 [54.6]	39 [54.2]	20 [55.5]
extensive (≥ 50 times)	21 [19.4]	19 [26.3]	2 [5.6]
Age of first snus experience, mean years (± SD)	17.3 (±1.9)	17.3 (±2.1)	17.2 (±1.1)

Values represent number of participants, except for current age, age of starting winter sport, and ages of first snus experience (years; mean ± SD). [%] = percentage of corresponding Participants column (Totals, Male or Female), except for [a] = percentage of Totals Participants value.

At the time of interview, 54 participants were snus users (50% of the total) with a higher prevalence among the group of males (58.3% out of the total of males) compared to the group of females (33.3% out of the total of females). Male gender is a risk factor for being current snus user (odds ratio = 2.800 [C.I. 1.213, 6.464]; p = 0.024). All females taking snus were occasional users (less than once use a day), whereas 11 out of 72 males were regular users (15.3% using snus once or more a day, vs. 43% of occasional snus users) (Table 2).

Table 2. *Survey I. STUDY I. Prevalence of snus and smoke use.*

	Totals [%]	Male [%]	Female [%]
Participants, *N*	108	72	36
No current snus users, *N*	54 [50.0]	30 [41.7]	24 [66.7]
Current snus users, *N*	54 [50.0]	42 [58.3]	12 [33.3]
occasional	43 [39.8]	31 [43.0]	12 [33.3]
regular	11 [10.2]	11 [15.3]	0 [0.0]
No current smokers, *N*	67 [62.0]	49 [68.0]	18 [50.0]
Current smokers, *N*	41 [38.0]	23 [31.9]	18 [50.0]

Values represent number of participants. [%] = percentage of corresponding Participants column (Totals, Male or Female).

Forty-one participants out of 108 were current smokers (38%) with a trend of gender prevalence opposite to that reported for snus: 50% of females vs. 31.9% of males were current smokers (odds ratio = 0.4694 [C.I. 0.2067, 1.066]; NS). The average number of smoked cigarette/day is low (4.4 ± 3.9 SD) with no difference between genders.

In Survey II among 61 participants, there were more males than females (51 vs. 10) (Table 3). The mean age was 26 years (± 5.4, SD). Fifty-five participants practiced alpine skiing, three snowboarding, two nordic skiing and only one curling. Thirty-three participants were agonistic athletes (54% of the total), with all of them began activity at 4.9 (± 3.6), years of age (± SD). Fifty-two participants (85.2%) practiced more than one exercise or sport (e.g., cycling, soccer) other than the main sport activity (Table 3).

Table 3. *Survey II. STUDY I. Demographic of participants*

	Totals [%]	Male [%]	Female [%]
Participants, *N*	61 [100]	51 [83.6][a]	10 [16.4][a]
Age in years, mean (± SD)	26 (±5.4)	26.1 (±5.5)	25.9 (±5.7)
Athletes performing agonistic winter sports, *N*	33 [54.0]	29 [87.8]	4 [40.0]
alpine skiing	55 [90.2]	48 [94.1]	7 [70.0]
snowboard	3 [4.9]	3 [5.9]	0 [0.0]
nordic skiing	2 [3.3]	0 [0.0]	2 [20.0]
curling	1 [1.6]	0 [0.0]	1 [10.0]
Age of starting winter sport, mean years (± SD)	4.9 (±3.6)	4.7 (±3.2)	5.6 (±5.4)
Athletes performing other exercise or sport activity, *N*	52 [85.2]	44 [86.3]	8 [80.0]

[%] = percentage values of corresponding Participants column (Totals, Male or Female), except for [a] = percentage of corresponding Totals Participants value.

At the time of the survey, 49 athletes were currently using snus (80.3% of the total). Among them twenty-five subjects were occasional snus users (19 male and 6 female) and 24 were regular snus users (21 male and 3 female) (Table 4)

Thirty-two athletes were current smokers (52.5% out of the total) with 27 males (53% out of the total of males) and 5 females (50% out of the total of females). Twenty-nine participants out of 61 were current smokers (47.5%) with 24 males (47% out of the total of males) and 5 females (50% out of the total of females) as shown in Table 4.

Table 4. *Survey II. STUDY I. Prevalence of snus and smoking use.*

	Totals [%]	Male [%]	Female [%]
Participants, *N*	61	51	10
Age of first snus experience in years, mean (± SD)	20.3 (± 5.7)	19.8 (± 5.7)	22.5 (± 4.7)
No current snus users, *N*	12 [19.7]	11 [21.6]	1 [10.0]
Current snus users, *N*	49 [80.3]	40 [78.4]	9 [90.0]
occasional	25 [41.0]	19 [37.2]	6 [60.0]
regular	24 [39.3]	21 [41.2]	3 [30.0]
No current smokers, *N*	32 [52.5]	27 [53.0]	5 [50.0]
Current smokers, *N*	29 [47.5]	24 [47.0]	5 [50.0]
occasional	17 [27.8]	14 [27.4]	3 [30.0]
regular	12 [19.7]	10 [19.6]	2 [20.0]

Values represent number of participants. [%] = percentage values of corresponding Participants column (Totals, Male or Female).

The average of mCEQ score for each item reported by occasional and regular current snus users is shown in Table 5. All mean mCEQ score values for each item were significantly different from score value = 1 (“Not at all”), thus confirming that snus use induced reinforcing effects. The existence of a dose-effect relationship between snus use and reinforcing effects was tested by comparing mCEQ score values for each item in occasional vs. regular users. As show in Figure 4 and in Table 5, a significant difference in mCEQ scores between the two levels of snus use was observed only for three items, that is item 1 “Was using snus satisfying?”, item 2 “Did snus taste good?” and item 12 “Did you enjoy using snus?”, suggesting that the existence of a dose-effect relationship was limited to few items.

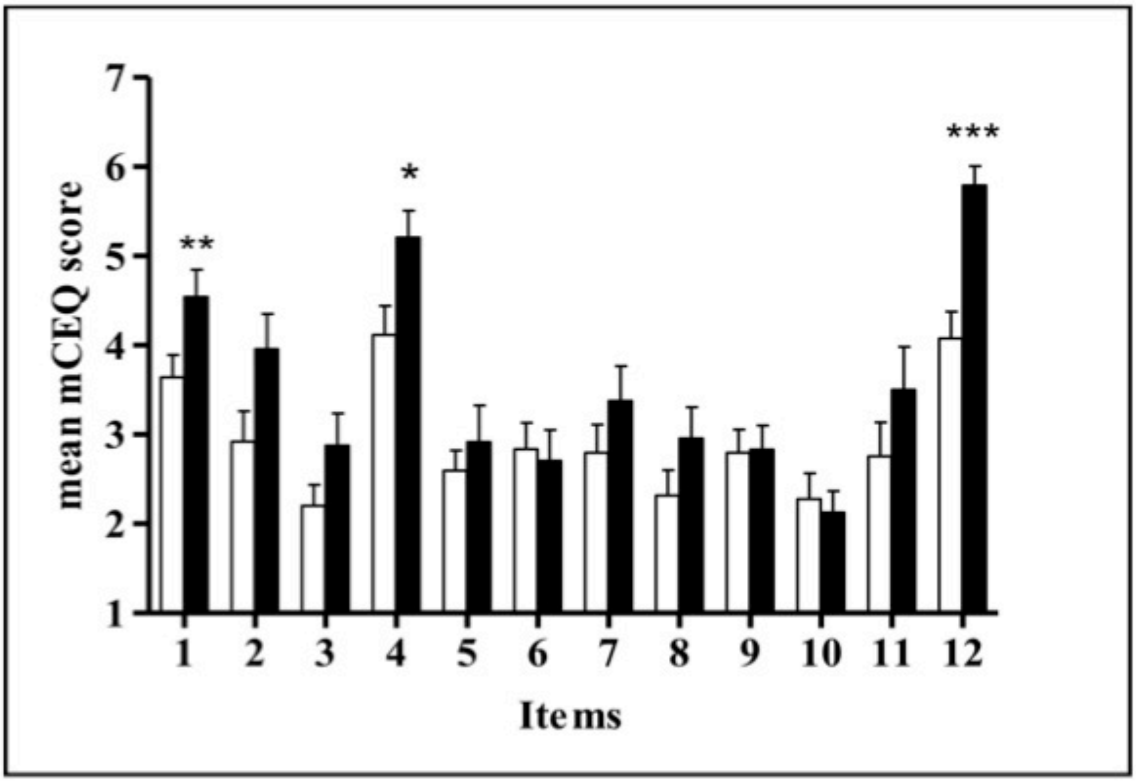

*Figure 4. Survey II. STUDY I. Score values (mean, SE) for each mCEQ item in occasional and regular current snus users. Bars represent occasional (open) and regular (solid) snus users. Items 1 to 12 respectively represent: 1 "Was using snus satisfying?", 2 "Did snus taste good?", 3 "Did you enjoy the sensations in your throat and chest?", 4 "Did using snus calm you down?", 5 "Did using snus make you feel more awake?", 6 "Did using snus make you feel less irritable?", 7 "Did using snus help you concentrate?", 8 "Did using snus reduce your hunger for food?", 9 "Did using snus make you dizzy?", 10 "Did using snus make you nauseous?", 11 "Did using snus immediately relieve your craving for a cigarette?", 12 "Did you enjoy using snus?". *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, unpaired Student t-test comparison between occasional current snus users versus regular current snus users for each item.*

Table 5. *Survey II. STUDY I. Mean (SE) score value for mCEQ items in occasional and regular current snus and smoke users.*

mCEQ item	Occasional snus users (*N* 25)		Regular snus users (*N* 24)		p value	Occasional smoke users (*N* 17)		Regular smoke users (*N* 12)		p value
1	3.6	(0.2)	4.5	(0.3)	0.0088**	3.0	(0.4)	4.6	(0.4)	0.0084**
2	2.9	(0.3)	4.0	(0.4)	0.0583	2.4	(0.3)	4.8	(0.4)	0.0003***
3	2.2	(0.2)	2.9	(0.4)	0.2521	2.4	(0.3)	4.2	(0.4)	0.0045**
4	4.1	(0.3)	5.2	(0.3)	0.0252*	3.6	(0.4)	5.2	(0.3)	0.0094**
5	2.6	(0.2)	2.9	(0.4)	0.9600	2.0	(0.3)	3.5	(0.4)	0.0063**
6	2.8	(0.3)	2.7	(0.3)	0.6515	2.7	(0.3)	4.1	(0.5)	0.0177*
7	2.8	(0.3)	3.4	(0.4)	0.2968	1.9	(0.2)	3.8	(0.4)	0.0007***
8	2.3	(0.3)	3.0	(0.3)	0.1663	3.6	(0.5)	3.6	(0.5)	0.9329
9	2.8	(0.3)	2.8	(0.3)	0.8881	2.5	(0.4)	1.5	(0.2)	0.1339
10	2.3	(0.3)	2.1	(0.2)	0.8482	1.9	(0.3)	1.3	(0.1)	0.0831
11	2.8	(0.4)	3.5	(0.5)	0.2522	3.9	(0.5)	5.0	(0.6)	0.1750
12	4.1	(0.3)	5.8	(0.2)	0.0001***	3.1	(0.4)	5.0	(0.4)	0.0012**

**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *unpaired Student's t-test comparison between occasional current users versus regular current users for each item.*

On the other hand, a significant difference between occasional and regular smokers was observed for a greater number of items, that is for items 1, 2, 3, 4, 5, 6, 7 and 12 (see Table 5), suggesting that the quantitative subjective description of smoking – induced reinforcing effect could be discriminated depending to the dose (i.e., amount of use).

According to Cappelleri et al (2007) groups of items could be clustered into five psychological domains (see Methods). As shown in Table 6 mean mCEQ score values where similar across domains (except for Aversion in regular smokers) thus suggesting that snus use – as well as smoking – induced reinforcing effect equally distributed across domains. Interestingly, the only significant differences for items 1, 4, and 12 between occasional and regular snus use (see above) correspond to the Satisfaction - items 1 and 12 and Psychological reward - item 4.

Table 6. *Survey II. STUDY I. Mean score (SE) mCEQ score values pooled into five domains for current snus users and smokers.*

Domain	Current snus user		Current smoke users	
	Occasional	Regular	Occasional	Regular
Satisfaction	3.5 (0.3)	4.8 (0.3)	2.8 (0.2)	4.8 (0.1)
Psychological reward	2.9 (0.3)	3.4 (0.5)	2.8 (0.4)	4 (0.3)
Enjoyment of respiratory tract sensations	2.2 (0.2)	2.9 (0.4)	2.4 (0.3)	4.2 (0.4)
Craving reduction	2.8 (0.4)	3.5 (0.5)	3.9 (0.5)	5 (0.6)
Aversion	2.5 (0.3)	2.5 (0.4)	2.2 (0.3)	1.4 (0.1)

Satisfaction = items 1, 2, and 12; Psychological Reward = items 4, 5, 6, 7 and 8; Enjoyment of Respiratory Tract Sensation = item 3; Craving Reduction = item 11; Aversion = items 9 and 10.

2.4 Discussion

This report describes an unexpected phenomenon of snus use in a sample of winter sport athletes in Italy. This segment of snus user population was characterized by healthy, young athletes performing more than one sport, full-time involved in training and competitive winter sport activities (mostly Alpine and Nordic skiing) since the time of their childhood. Moreover, the survey was conducted in an European country where there is not socio-cultural tradition and marketing of snus. When a different sample of winter sport athletes were asked to rate snus reinforcing effects (Survey II), it emerged that snus use induced reinforcing effects similar to smoking tobacco, and that some of these effects – those associated to reward and satisfaction - were positively related to snus daily intake (occasional vs. regular).

On Survey I, an average of three-quarter of the participants reported past experience with snus. Fifty-five participants were current snus users, most of them with an occasional pattern of administration (less than once a day), whereas about 10% of the participants were regular users. Data from Survey I were also confirmed in Survey II: as expected the prevalence of current snus users was higher in the latter (80.3 vs. 50%) because the participants selection criteria (to have a past or current status of snus user).

This prevalence among an athlete population is not surprising. Since ’80s and ‘90s, anecdotal and literature reports showed that young people involved in sport activities were users of smokeless tobacco (Glover et al., 1986; Glover et al., 1988; NCAA 1989). U.S. surveys among college athletes reported prevalence of smokeless tobacco use ranging between 30 and 40% (Connolly et al., 1988; Ernster et al., 1990; Severson et al., 1998). Walsh and colleagues (Walsh et al., 1994; Walsh et al., 2000) have extensively investigated the phenomena of spit tobacco among baseball players. Interestingly, Davis et al., (1997) showed that smokeless tobacco use among high school male students was correlated to athletic activity, and that sport participation was a predictor of such as use. To our knowledge, published reports about snus use among winter sport athletes are few (Bujon 2008; Schweizer et al., 1998). A recent analytical chemistry study confirmed that ice hockey players take relevant amounts of nicotine through smokeless tobacco (Marclay and Saugy 2010). Marclay and colleagues (2011), by using analytical chemistry detection, assessed smokeless tobacco use in athletes from various sports by measuring nicotine and metabolites levels.

Our survey was limited by the small sample size, due to the limited dimension of this specific segment of the population. The number of participants that agreed to be interviewed was also limited by the fact that the survey was conducted in proximity of competitions when some athletes refused to participate because the concomitant sport event.

On Survey II, the findings showed that snus induced a series of reinforcing effects. All the data were significantly different from score value = 1, that is “Not at all” effect. It was found interesting to test the possible relationship between mCEQ score value and snus daily use. The comparison between the two levels of intake (occasional vs. regular snus use), showed a significant difference for items 1, 4 and 12. Interestingly, these items account to Satisfaction (items 1 and 12) and Psychological reward (item 4) domain sub-scales. Satisfaction and Psychological reward subscale were those with higher reliability among the five domains, and were moderately and positively correlated (Cappelleri et al., 2007).

The inclusion in Survey II of the same assessment for reinforcing effects of smoking gave an indicative comparison between snus and smoking effects. It is evident that although occasional smokers did not report score values significantly different from occasional snus users, regular smoking induced however a greater reinforcing effect as it is shown by higher mCEQ scores. Significant differences between occasional and regular smoking score values were shown for 8 out 12 mCEQ items vs. 3 out of 12 for snus. Although both snus and smoking is a delivery of nicotine, the degree of nicotine dependence was not measured in our study. It will be therefore important to further explore the nicotine dependence component of snus use in these participants, by also taking in consideration environmental factors (e.g., the sport context) in order to build a more comprehensive hypothetical framework that could explain snus use in sports.

There are currently unanswered questions, such as the nicotine dependence degree, and about the interaction between nicotine effects and the psycho-physiological performance under competition. According to previous literature data, it could be speculated that socio-cultural and/or psychobiological factors plays a role in initiation and maintenance of this habit. Italian winter sport athletes are involved in a series of winter sport competitions at a closer contact with a successful role-model (Wiium et al., 2009; Gottlieb et al., 1993), such as that offered by those North American and North European athletes using snus. It is also possible that the sport environment in general may facilitate the availability of snus, as reported in previous studies (Huhtala et al., 2006; Karvonen et al., 1995).

In conclusion, this survey confirms a high prevalence of snus use among winter sport athletes. At present, there are no data that could warn against such as use as detrimental for sport performance, or as a form of doping. On the other hand, risks associated to snus-contained carcinogenics are well-known (ESTOC 2008). Although there is consensus that the health risks of smoking are much greater than those of snus, it is however important to increase information and education among this segment of young athletes.

3. STUDY II

Effects of snus administration on exercise endurance in men

3.1 Introduction

Snus is a smokeless tobacco consumed orally, not smoked, traditionally produced and used in Sweden (Rutqvist et al., 2011). It is typically placed between the upper lip and the gum for approximately 30 min before it is discarded. Sweden is the only country in the EU granted special exemption to manufacture and snus sales (Anon 1992; Ahlbom et al., 2007; Fagerström and Schildt 2003*).* In 2006, snus was introduced in USA and Alpert and colleagues (2008) reported a increasing trends in its use.

Zandonai et al (2013) reported an increase snus use among alpine skiers in northern Italy. An analytical chemistry study confirmed that ice hockey players take relevant amounts of nicotine through smokeless tobacco (Marclay and Saugy 2010). More recently, Marclay and colleagues, by using analytical chemistry detection, assessed smokeless tobacco use in athletes from various sports by measuring nicotine and metabolites levels (Marclay et al., 2011). On 2012, nicotine has been placed on WADA's 2012 Monitoring Program (WADA 2012) in order to detect potential patterns of abuse.

There are however unanswered questions about sought and subjective reinforcing effects experienced by this specific athletic population. In fact, from a psychobiological standpoint, snus is a delivery of a psychoactive and addictive substance, nicotine (Henningfield et al., 1997; Fagerström et al., 2011).

Only few experiments have been performed to test human performance under the effect of smokeless tobacco, and none on snus. In particular, Mundel and Jones (2006) have shown that nicotine administered through the skin (patch), increased the duration of an endurance performance but not the perception of fatigue when compared to a placebo condition. Authors concluded that nicotine prolonged the endurance performance throughout a central mechanism. Indeed, there are different ways to measure the effect of fatigue at a cognitive-perceptual level: one is to consider the subjective intensity of effort, strain, discomfort, experienced during a physical exercise (Robertson and Noble 1997) whereas the other is the neuro-physiological discrimination from two different sources, one peripheral and one central (Enoka and Stuart 1992; Gandevia 2001).

Therefore, the aim in this study to investigate the snus effects on exercise endurance correlated with the perception of fatigue along with a range of physiological and neuro-physiological parameters. We predict that administration of snus during exercise at 65% W_{max} until exhaustion, will confirm Mundel and Jones's results described above.

3.2 Materials and Methods

3.2.1 Participants

We recruited 14 healthy males (18 to 65 years) volunteers non-smokers and non-snus users. The exclusion criteria were the presence of symptomatic cardiopathy, metabolic disorders such as obesity (BMI >30) or diabetes, chronic obstructive pulmonary disease, epilepsy, therapy with beta-blockers and medications that would alter cardiovascular function, hormonal therapy, alcoholism and smoking.

3.2.2 General Design

All participants were submitted to a medical visit (MD) and they were informed about the procedures and risks and they signed an informed consent form at the end of the MD.

The institutional review board of the Department of Neurological, Neuropsychological, Morphological and Motor Sciences, University of Verona, approved the study protocol, the experimental design and methods that conformed to the 1964 Declaration of Helsinki.

The protocol consists of three experiments carried on an electrically braked cycle-ergometer (Sport Excalibur, Lode, The Netherlands) set in the pedal rate independent mode. Experiment 1 (EXP1): an incremental exercise test to determine maximal aerobic power output (W_{max}). Experiment 2 (EXP2) and experiment 3 (EXP3) involve exercising at 65% W_{max} until exhaustion and Swedish snus (SS) or Swedish snus placebo (SP) was administered to participants at the start exercise (EXP2 and EXP3). Before and after all experiments participants were administered the Profile of Mood of State questionnaire (POMS) and tested with Transcranial Magnetic Stimulation (TMS) to check changes in cortico-motor excitability due to the prolonged exercise. The study was a double-blind crossover design study comparing the effect of SS vs.

SP on exercise endurance. A MD was conducted in the same day of the EXP1 and after three days all participants were attended EXP2. EXP3 began at least one week apart EXP2. Participants were randomized to blindly receive SS or SP on EXP2 day and on EXP3 day.

3.2.3 Protocol

Participants arrived at the School of Exercise and Sport Sciences, University of Verona, by 08.30 a.m., wearing cycling shorts and socks.

LAP (Laboratory Action Perception) session. Participants were accompanied into the LAP where it was administered the POMS questionnaire. Participants read the questions on the monitor and answered with the aid of a numeric keyboard. Then, the TMS session was carried out. The optimal scalp position (OSP) over the left primary motor cortex for eliciting motor evoked potentials (MEPs) was selected to record stable MEPs from two lower leg muscles (Tibialis Anterior (TA) and Vastus Lateralis (VL) (First dorsal Interroseous (FDI) as a control)). During the recording session the coil position was over the left motor cortex, in correspondence with the OSP. The resting motor threshold (rMT) was defined as the lowest stimulus intensity able to evoke 5 out of 10 MEPs with amplitude of at least 50 μV in the VL. Twenty transcranial stimuli (TMS20) were delivered to the relaxed muscle at a stimulus intensity of 1.2 × motor threshold (MT), in order to investigate MEP characteristics such as peak-to-peak amplitude and latency at a standardized intensity (Verin et al., 2004). After these measurements we connected S-Beam Load Cell (DBBSE), securely strapped into the floor, to the dominant limb ankle to maximum voluntary contraction (MVC). Participants were performed six brief (3s) control MVCs of the Vastus Lateralis (VL), from which peak torque and peak surface EMG was measured. Six peripheral stimuli were then delivered to the relaxed muscle.

Exercise Physiology Lab session. EXP1. The participants performed on a cycle-ergometer the first incremental sub maximal test, in order to get a considerable workload-V'O2 relation. The incremental ramp exercise protocol, preceded by 6-min of rest, consisted in cycling on a workload which increased by 30 W every 6 min. The initial workload was 0W and the last step was 150W, pedal frequency was held constant at 75 rpm. After 30 min of rest and rehydration a second test was performed, as reported below.

The incremental exercise test workload initial was 50 W, which increased by 25 or 35 W every 1 min until voluntary exhaustion. The latter was defined as the inability to maintain the pedaling frequency (60 – 80 revolutions/min (rpm)), despite vigorous encouragement by the experimenters. Heart rate (HR) was monitored continuously as well as expiratory minute ventilation ($\dot{V}E$), O2 consumption rate ($\dot{V}O2$) and rate of CO2 production ($\dot{V}CO2$), from which the respiratory exchange ratio (RER). The test was considered maximal if one of the following criteria was met: (1) final HR was within 10% of predicted maximum; (2) a clear plateau in oxygen uptake was noticed; or (3) respiratory exchange ratio will be equal to, or above, 1.10 (ACSM 2009). Global rating of perceived exertion (RPE) was recorded in the last 15 s of each exercise new incremental step and was recorded using the 15-point Borg scale (Borg 1982a). Maximal aerobic power output was identified as the intersection point between individual relations of V'O2 to W' (from the first test) and the V'O2 max value (from the second test).

At the end of the EXP1, participants were accompanied into the LAP for TMS20 and MVC experiments and POMS questionnaire, in this order.

EXP2 and EXP3. Participants were asked to record, in a specific table sent by mail, their diet in the 24 h period before the EXP2 and were instructed to follow the same diet before each subsequent experiment. They had to abstain from physical activity, alcohol and caffeine consumption. The measurement of exhaled carbon monoxide (CO) level was performed using the EC50 Micro Smokerlyzer† (Bedfont Scientific Ltd.). During the exercise at 65% W_{max} until exhaustion were continuously measured: i) stroke volume of the heart and cardiac output by means of cardio-impedance; ii) pulse pressure profile, mean, systolic, diastolic pressures by means of photoplethysmography: iii) muscle and cerebral oxygenation by means of NIRS (near-infrared spectroscopy) (Perrey 2008). The muscular NIRS lightweight plastic probe was longitudinally positioned on the belly of the VL muscle 15 cm above the patella and attached to the skin with a bi-adhesive tape. The cerebral NIRS lightweight plastic probe was positioned on the frontal side, the same of the dominant leg, and attached to the skin. Arterial pressure profile was continuously recorded at a fingertip by using a non-invasive photoplethysmographic method. Participants remained stationary on the cycle-ergometer for 5 min to acquire baseline measurements. Blood samples were taken at time -3 min to determine the blood lactate concentration ([La]b mM), blood glucose concentration ([G]b mM) and ([Hb]b, g). In the last 10 sec they took the sachets of SS or SP from the box. They placed in the anterior part of mouth between the upper gingiva slightly pulling the mask (Time zero = T0). The participants had to keep in their mouth the SS or SP until the end of the exercise. Immediately after they started pedaling with a frequency around 75 rpm/min for 5 min (warm up). The load in warm-up was (means ± SD) of 100 ± 50 W and it was calibrated on EXP1 for each subject. Global rating of perceived exertion (RPE) was recorded in the last 15 s of each 5 min until the end of the test. RPE was recorded using the 15-point Borg scale (Borg 1982). Blood samples to determine the blood lactate concentration ([La]b mM), blood glucose concentration ([G]b mM) and ([Hb]b, g) were taken each 10 min from T0 until the end of the test. At the end of warm-up started the exercise at 65% W_{max} until exhaustion with a load calibrated on EXP1 for each subject. In figure 5 a schematic representation of the protocol and its measurements.

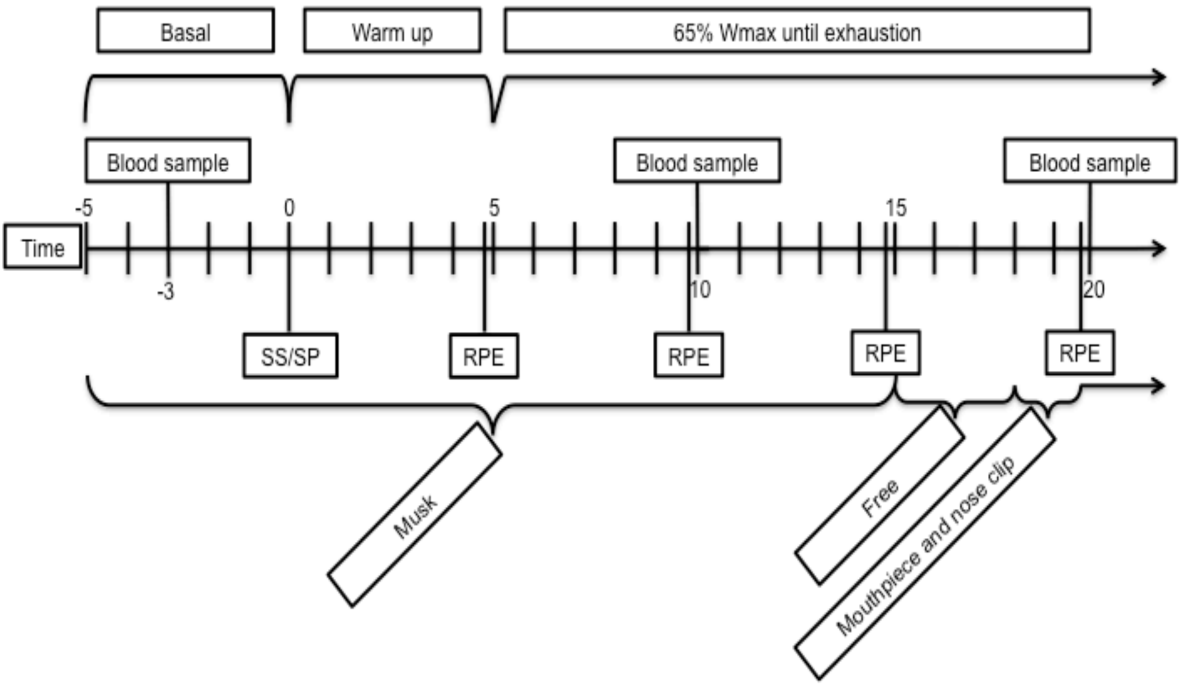

Figure 5. STUDY II. Schematic representation of the protocol and its measurements in minutes. Abbreviations: SS/SP: Swedish snus or Swedish snus placebo; RPE: Rating perception of effort (Borg's scale).

At the end of the exercise at 65% W_{max} participants spat out snus. Immediately we performed an interview to participants about nicotine adverse events (if yes: mild / moderate/ serious). Participants were taken 5 ml of blood to nicotine and cotinine measurements. At the end of the physiological lab sessions the participants were accompanied into the LAP where were administered TMS20 and MVC experiments and POMS questionnaire in this order.

3.2.4 Materials

Snus

Swedish snus according to the GothiaTek standard is a low-nitrosamine, moist oral tobacco product with water content of approximately 45%–55% and a pH of approximately 8.5. Lunell and Lunell (2005) described nicotine delivery and uptake from snus sachets. The mean time to maximum nicotine plasma concentration (Tmax) is 37.1 ± 10.2 (SD) min (range: 24–60 min) for 1 g Swedish portion snus (Lunell and Curvall 2011). Fagerstrom et al (2012) show a lack of serious AEs that require medical intervention. The placebo was almost identical to the snus in physical appearance, mouth feel, pH, flavouring, and other sensory characteristics, but it did not contain tobacco or nicotine. The placebo is composed of water, oat and cocoa fibers, humectant (E422), flavor enhancer (sodium chloride), acidity regulator (E504, E500) and aromas. In this study were administrated a commercial Catch White Eucalyptus Portion Snus (Swedish Match) 1.0 g for SS and Onico Pepparmint (Swedish Match) 1.0 g for SP respectively.

POMS questionnaire

The POMS has been used extensively for the assessment of mood in the sport and exercise environments. This questionnaire has 58 items and the factor structure representing six dimensions of the mood construct: Tension, Depression, Anger, Vigor, Fatigue and Confusion. Items were answered on a 5-point Likert scale (0 = not at all, 1 = a little, 2 = moderately, 3 = quite a bit, 4 = extremely). Raw scores were transformed following the standard point table (McNair et al., 1991).

The DBBSE series S-beam load cell

The DBBSE series S-beam load cell (Applied Measurements Ltd., Berkshire, UK) is designed for force measurement and weighing applications alike. Characteristics' cell load are: capacities: 0-1kg up to 0-100; output: 2.0 ± 0.1mV/V; high accuracy: < ± 0.03%/RC. Force was transmitted, amplified, and displayed on a personal computer running Spike2 software, via a CED 1401 data logger (Cambridge Electronic Design Ltd., Cambridge, UK).

Transcranial Magnetic Stimulation (TMS)

TMS was performed by using a 70-mm figure-of-eight coil connected to a Magstim 200 Rapid (Magstim, Whitland, Dyfed, UK), producing a maximum output of 2 T at the coil surface (pulse duration, 250 msec; rise time, 60 msec). Muscle activity was recorded and amplified with a Digitimer D360 8-channel and CED Power 1401 (Digitimer, Hertfordshire, England), band-pass filtered (20 Hz–3 kHz) and sampled at 5 kHz. Electromyographic activity was recorded with surface electrodes over TA, VL and FDI muscles. TMS stimuli sequences were processed by the Psychology Tools' Eprime2 software (Pittsburgh, PA).

Maximal Voluntary Contraction

We have computed the MVC averaging 20 ms before the TMS pulse and we chose the best over six. We calculated the percentage of the condition post in relation to the condition pre. Moreover we measured the peak twitch tension after TMS and we averaged the 6 trial performed during the MVC and the 6 recorded at rest. We calculated the percentage of the condition post in relation to the condition pre. *TMS data*. For the TMS we computed the peak-to-peak amplitude of each MEP then, we eliminated the MEPs ± 2 standard deviations and finally, the average was computed.

Smoking status and cotinine level

The measurement of exhaled CO concentration, a non-invasive method of assessing smoking status, was measured using the EC50 Smokerlyser (Bedfont Instruments; Kent, UK). Bedfont EC50 analyzer is reported to correlate closely with blood carboxyhaemoglobin concentration in smokers and in non-smokers. The Smokerlyser measures breath CO levels in parts per million (ppm) based on the conversion of CO to carbon dioxide (CO2) over a catalytically active electrode (Middleton and Morice 2000). Venous blood sample (5 ml) were collected into prechilled EDTA-containing tubes. The blood was centrifuged at 2300 g for 12 min at 4°C and the plasma separated and stored at -20° C for nicotine and cotinine analysis.

Chemical and reagent

Nicotine, cotinine and acetanilide (internal standard: IS) were purchased from Sigma-Aldrich (Milan, Italy). Acetonitrile, dichloromethane, sodium hydroxide and ammonium acetate (Merck KgaA - Darmstadt, Germany); formic acid and hydrochlorid acid (Sigma-Aldrich - Milan, Italy) were analytical-reagent grade, milliQ water was filtered and deionised with Ultra Pure Water System, MilliQ-plus (Millipore, USA).

Chromatographic analysis

Nicotine, cotinine and acetanilinide (IS) were determined by means of an HPLC technique coupled with MS detection. Analytes were separated on a Restek Ultra-C8 column (150 mm X 2.1 mm - 5 μm) (USA) at a flow-rate of 0.2 ml min^{-1}. Separation was carried out in isocratic conditions with a solution of acetonitrile/water/formic acid + 2 mmol l^{-1} ammonium acetate (78/22/0.1 v/v). Chromatographic equipment consists of High Performance Liquid Chromatography (HPLC) LC-200 pump; samples were detected in *Selected Ion Monitoring (SIM)* (m/z nicotine m/z 163.1, cotinine 177.1 and acetanilide 136.0) with positive electrospray ionisation (ESI), by a Q-trap LC/MS/MS Systems (MDS Sciex - Ontario, Canada). Data were acquired and processed with *Analyst* 1.4. (Applied Biosystems package, MDS Sciex - Ontario, Canada). The system operated at room temperature. In these conditions nicotine, cotinine and acetanilide retention times were 2.38, 2.45 and 2.78 minutes respectively.

Calibrators and quality control samples

Calibrators and control samples containing nicotine and cotinine were prepared adding known amounts of analytics to blank serum. They were included in each batch of volunteers' samples. Calibration curves and quality control samples ranged respectively, from 2 to 50 and 3 to 15 ng ml^{-1} for nicotine and from 20 to 500 and 30 to 150 ng ml^{-1} for cotinine. Limit of detection (LOD) for nicotine and cotinine were 2 and 20 ng mL^{-1} respectively.

Sample preparation

To purify nicotine and cotinine from serum samples, we modified the extraction procedure proposed by Nakajima et al (2000); extraction was a simple two steps procedure: alkalinisation and organic extraction. One ml of serum was transferred to a polypropylene tube, followed by addition of 50 µl of internal standard (acetanilinide 10 ng $µl^{-1}$) and 50 µl of sodium hydroxide (10M). The tubes were vortexed and samples extracted with 8 ml of dichloromethane by rotation for 10 minutes and centrifuged at 3000 RPM for 10 minutes. The organic layer was transferred to a glass tube containing 50 µl of hydrochlorid acid (37%). After evaporation of the organic phase, under a nitrogen stream at 40°C, the residue was dissolved with 75 µl of mobile phase and 20 µl was injected into the HPLC system.

Physiological measurements

Breath by breath gas exchanges and heart rate (HR) were measured using metabolic cart (Quark b2, Cosmed, Italy) during all experiments. NIRS is a non-invasive technology that makes it possible to continuously monitor the changes (either relative or absolute) in oxy- (O2Hb), deoxy- (HHb), total Hb (THb) and myoglobin (Mb) concentrations, during a dynamic exercise (Mancini et al., 1994). Heart stroke volume (SV) was determined on a beat-by-beat basis by means of the Modelflow method, applied off-line to the pulse pressure profiles using the Beatscope software (Portapres, FMS, Amsterdam, The Netherlands).

Blood sample

The blood lactate concentration ([La]b, mM) and blood glucose concentration ([G]b, mM) in arterialised capillary blood was assessed by means of an electro-enzymatic method (Biosen C_line, EKF Diagnostic, Barleben, Germany) on 10 ll blood samples taken from an earlobe. The blood hemoglobin concentration ([Hb]b, g) in arterialized capillary blood was assessed by means of HemoCue® Hb 201+, Hemocue AB, Ängelholm, Sweden.

Data analysis

Times to exhaustion data were analyzed using a paired Student's t-test (SS vs.

SP). RPE data were analyzed using a paired Student's t-test (SS vs. SP). POMS data were analyzed using ANOVA two-way test (pre/post – SS/SP). For the MVC, muscle twitch and TMS were analyzed using a full within ANOVA test (SS vs. SP). Then, to compare the condition post with the condition pre an one sample t-test was used with an alternative hypothesis not equal to 100. The normality and homogeneity of the variance were tested using the Shapiro-Wilk normality test and the Bartlett test of homogeneity of variances. For the data that did not have homogeneity of variance, the data were log transformed and a Welch one-way ANOVA was used. Statistical significance will be accepted at $P < 0.05$.

3.3 Results

Participants

Fourteen participants were recruited and one subject withdrew from the study for a leg injury at the end of EXP2. Participants' characteristics were (means ± SD): age, 22.8 ± 4.6 years; height 177.3 ± 6.1 cm; $VO_{2\ max}$, 48.9 ± 6.4 ml kg^{-1} min^{-1}; W_{max}, 205.7 ± 46.2 W; HR_{max}, 181 ± 9 beats min^{-1}. The weight in EXP1 was 73.9 ± 7 kg, while in SS test and in SP test was 74.1 ± 7 kg and 73.8 ± 7.2 kg respectively. Three participants practiced soccer, three running, two tennis and the remaining practiced gymnastic, gym, basket, mixed martial arts (MMA) and swimming.

Smoking status assessment and nicotine/cotinine levels

The level of CO (ppm) was (means ± SD) 0.9 ± 0.5 before to start SS test and 1 ± 0.9 before to start SP test. Values defined the participants as non-smokers, in accordance to Deveci et al (2004). The blood sampling has been carried out (time from participants spat out) (means ± SD): 5.2 ± 2.2 min for SS test and 6.4 ± 2.6 min for SP test from the end of exercise. Detected plasma nicotine level was 7.31 ± 1.78 ng/ml (means ± SD) in 7 participants for SS and 3.26 ± 0.12 ng/ml (means ± SD) in 3 participants for SP. In three participants nicotine and cotinine were no detectable.

Adverse events

Five participants reported adverse events at the end of SS experiment. One subject reported a mild discomfort in the throat and another one a mild nausea and dizziness. A moderate nausea and dizziness was highlighted from two participants.

Exercise capacity and perceived exertion

For all participants, the average time to exhaustion was 60.4 ± 41.5 min during SS session and 48.8 ± 19.4 min during SP session, a +18.6% increase but not significant at paired Student's t-test (P value 0.644) as show in Figure 6.

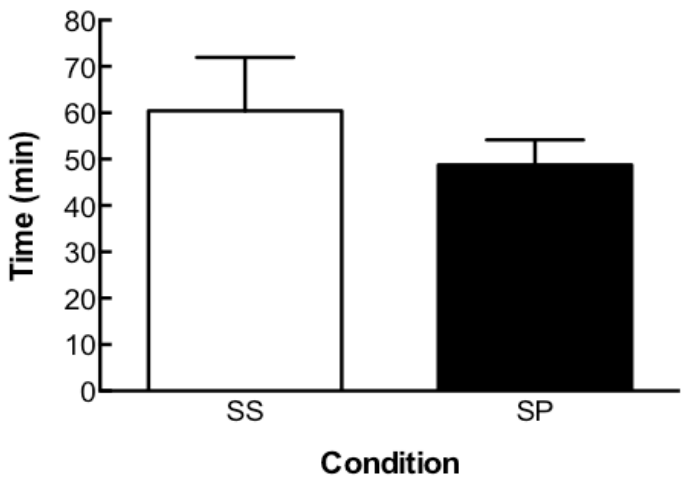

Figure 6. STUDY II. Mean time to exhaustion for all participants. Mean time to exhaustion (min; ± SD) in all participants during SS □ and SP ■ test (n=13).

Seven out 13 participants cycled for longer during the SS tests (figure 7).

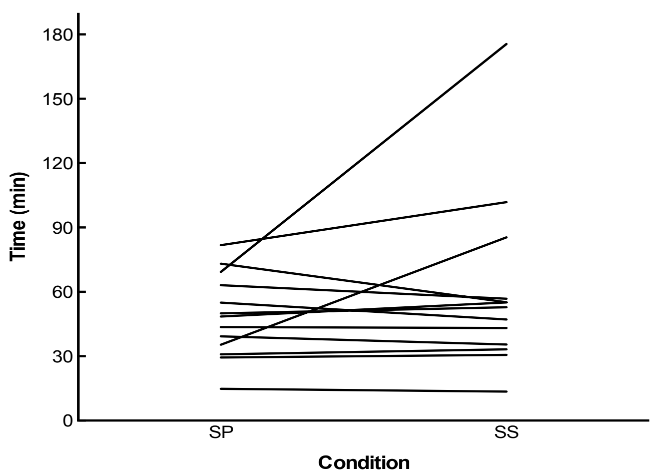

Figure 7. STUDY II. Exercise times (min) for individual subject during SS and SP test (n=13).

One subject was discarded because his time to exhaustion was insufficient to acquire four Borg scale values at least. RPE values at 25%, 50%, 75% and 100% time to exhaustion increased during both test; no differences were observed between each interval time in % and two conditions (placebo vs. nicotine) in paired Student's t-test (figure 8).

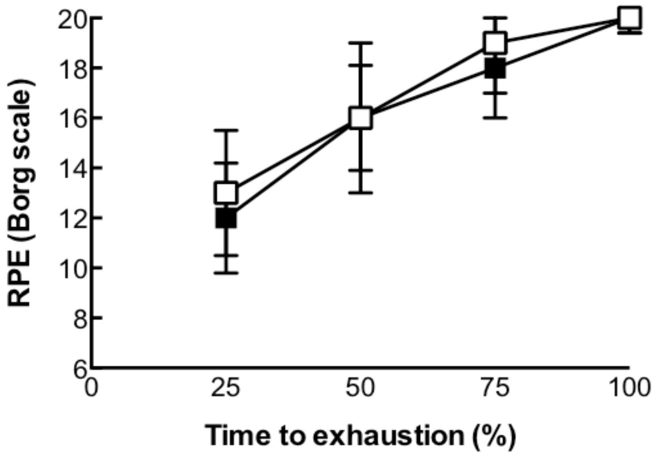

Figure 8. STUDY II. Borg scale values at 25%, 50%, 75% and 100% time to exhaustion. Borg values (mean ± SD) during SS □ and SP ■ test (n=13).

Perceived exertion (Borg scale values) showed no significant difference (P values 0.2031) in paired Student's t-test during the first 30 minutes of the time to exhaustion (figure 9).

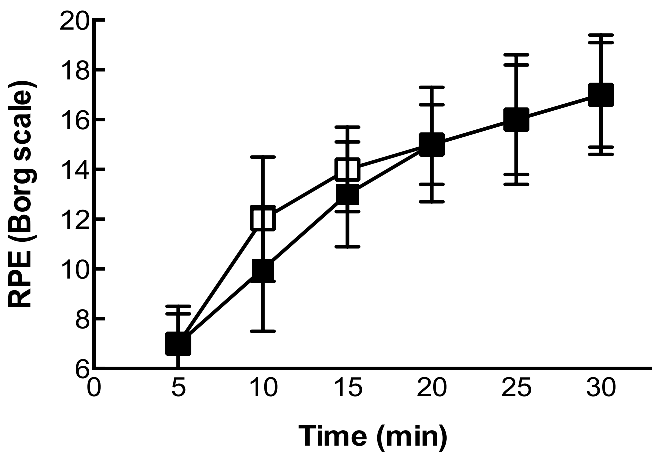

Figure 9. STUDY II. Borg scale mean values in the first 30 minutes time to exhaustion. Mean values (± SD) during exercise for SS □ and SP ■ test (n=12).

POMS

The POMS questionnaire was measured pre and post for all experiments. Table 7 show significant differences for Anger, Vigor and Confusion factors in pre and post administration for all experiments and Fatigue factor for SS; no differences were observed for Tension and Depression factors. In ANOVA two-way test (pre/post – SS/SP) no differences significant were observed.

Table 7. *STUDY II. Mean ± SD POMS scores in SS and SP condition.*

	SS			SP		
POMS factors	pre	post	p value	pre	post	p value
Tension	40.4 ± 3.8	39.5 ± 4.7	0.3661	40.3 ± 3.9	40.2 ± 4.7	0.7381
Depression	43.2 ± 3.2	43.2 ± 2.5	0.3828	42.8 ± 2.9	43.3 ± 2.4	0.2165
Anger	43.5 ± 5.8	47.2 ± 2.8	0.0103*	41.8 ± 2.2	48.2 ± 2.7	<0.0001***
Vigor	50.8 ± 8.1	33.4 ± 3.7	°<0.0001***	52.7 ± 7.5	33.5 ± 3.2	<0.0001***
Fatigue	45.5 ± 9.1	50.8 ± 6.8	0.0332*	45.9 ± 7.5	50.8 ± 6.2	0.0620
Confusion	43.2 ± 5.1	35.7 ± 3.6	0.0012**	42.3 ± 4.4	36.4 ± 3.1	0.0038**

*Pre: pre exercise test; post: post exercise test. *P < 0.05, **P < 0.01, ***P < 0.001, paired Student's t-test (° normally distribution) comparison between pre exercise and post exercise in each experiment for six factors.*

MVC response

The MVC was measured pre and post for all experiments and the data are reported in Table 8. The ANOVA did not show any difference between the SS and SP condition F (1,12) = 0.006, P = 0.937, while one-sample t-test showed a significant difference between the measurement pre and post in both conditions (SS t = -3.480, df = 12, P = 0.004 and SP t = -4.714, df = 12, P < 0.001; (Figure 10 left panel)).

The analysis of the muscle peak twitch generated by the TMS during the MVC did not show any difference between the SS and SP condition F (1,12) = 0.003, P = 0.956, while the one-sample t-test showed a significant difference between the measurement pre and post in both conditions. SS t = -3.661, df = 12, P = 0.003 and SP t = -5.092, df = 12, P < 0.001 (Figure 10 left panel).

The Welch one-way ANOVA of the muscle peak twitch generated by the TMS at rest did not show any difference between the SS and SP condition (Table 8), F (1,12) = 0.5691, P = 0.4618, while the one-sample t-test showed a significant difference between the measurement pre and post only in the SS conditions. SS t = 3.123, df = 12, P = 0.009 and SP t = 2.041, df = 12, P = 0.064 (Figure 10 left panel).

Table 8. *STUDY II. Mean ± SD of the MVC and TMS.*

	SS					SP				
	Mean ± SD			Δ (Pre-Post)		Mean ± SD			Δ (Pre-Post)	
MVC	87.00	±	13.5%	-13.0%	*	86.60	±	10.2%	-13.4%	*
PTMVC	86.60	±	13.5%	-13.4%	*	86.40	±	9.7%	-13.6%	*
PTREST	103.90	±	4.5%	3.9%		106.50	±	11.5%	6.5%	
MEPVL	86.50	±	38.7%	-13.5%	*	77.10	±	36.9%	-22.9%	*
MEPTA	103.10	±	51.7%	3.1%		93.70	±	45.3%	-6.3%	

In the table are reported the mean ± the standard deviation (SD) and the difference between the condition pre and post (Δ(Pre-Post)). * P<0.05

Abbreviations: MVC: maximal voluntary contraction, PTMVC: Peak twitch generated by the TMS during the MVC; PTREST: Peak twitch generated by the TMS at rest; MEPVL: Motor evocated potential Vastus Lateralis; MEPTA: Motor evocated potential Tibialis Anterior.

TMS response

The TMS has been applied pre and post for all experiments and the results are reported on the Table 8. The ANOVA of the VL MEPs did not show any difference between the SS and SP condition F(1,11) = 0.505, P = 0.492. One sample t-test showed none significant difference between the measurement pre and post in the SS condition t = -1.206, df = 11, P = 0.253 and close to be significant in the SP condition t = -2.152, df = 11, P = 0.054 (Figure 10 right panel).

The analysis of the TA data did not show any difference between the SS and SP condition F(1,2) = 0.218, P = 0.650. Moreover, also the one-sample t-test did not reveal a difference between pre and post condition. SS t = 0.207, df = 11, P = 0.840 and SP t = -0.482, df = 11, P = 0.640 (Figure 10 right panel). The FDI as control muscle did not show any activation after TMS.

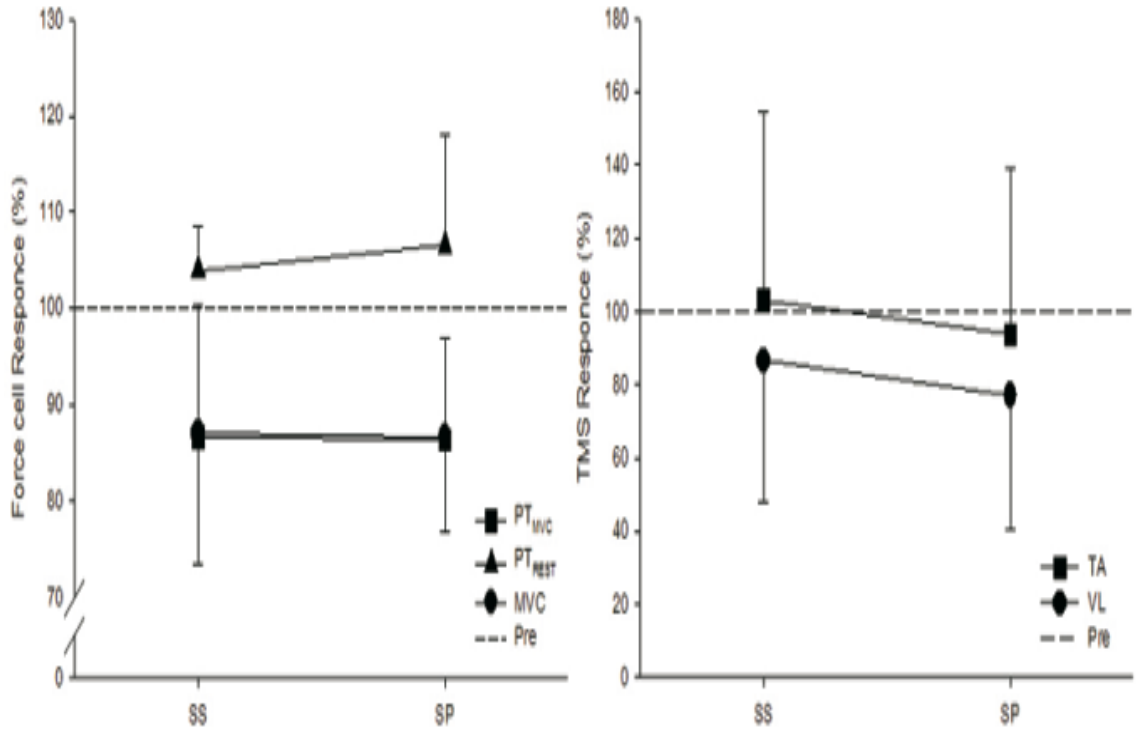

Figure 10. STUDY II. Force cell and TMS values in SS and SP condition. The panel on the left shows the results obtained by the force cell in the condition SS and SP and the variation respect to the condition Pre (dash line) of the maximal voluntary contraction (MVC), Peak twitch generated by the TMS during the MVC (PT_{MVC}) and the Peak twitch generated by the TMS at rest (PT_{REST}). The panel on the right shows the results of the TMS in the condition SS and SP and the variation respect to the condition Pre (dash line) of the muscle Vastus Lateralis (VL) and Tibialis Anterior (TA).

Cardiovascular and respiratory responses

Metabolic and cardiovascular results are reported at 25%, 50%, 75% and 100% time to exhaustion in Table 9 for thirteen participants. Values are slightly modified during both test; no differences were observed between the two conditions (SP vs. SS) in paired Student's t-test. Mean arterial pressure (MAP) at exhaustion decrease significantly in SS, $P < 0.05$.

Table 9. *STUDY II. Metabolic and cardiovascular results at 25%, 50%, 75% and 100% time to exhaustion*

Metabolic parameters	Condition	25%	50%	75%	100%
V̇E	SS	76.63±17.51	81.37±21.26	87.15±25.04	93.17±26.43
	SP	74.95±22.56	84.22±24.31	87.09±26.55	89.84±26.64
V̇O_2	SS	2.63±0.52	2.73±0.47	2.80±0.50	2.85±0.42
	SP	2.62±0.57	2.79±0.60	2.85±0.61	2.92±0.55
V̇CO_2	SS	2.77±0.50	2.79±0.42	2.79±0.45	2.75±0.33
	SP	2.76±0.54	2.83±0.53	2.80±0.55	2.82±0.47
RER	SS	1.06±0.05	1.03±0.05	1.00±0.04	0.97±0.04
	SP	1.06±0.05	1.02±0.05	0.99±0.04	0.97±0.05
Cardiovascular parameters					
MAP	SS	114.49±13.90	109.90±7.34	105.14±8.15	96.76±10.02*
	SP	110.94±16.23	106.09±13.99	108.71±8.48	108.47±8.80
HR	SS	136.89±25.65	157.25±13.37	162.26±11.21	161.68±12.72
	SP	124.70±30.75	150.42±14.09	159.18±10.24	161.80±10.85
C Ȯ	SS	17.53±3.80	20.19±2.70	19.59±2.50	17.97±3.20
	SP	15.15±4.59	18.07±4.28	19.19±2.36	18.84±2.94
TPR	SS	0.42±0.13	0.34±0.06	0.33±0.06	0.34±0.06
	SP	0.49±0.16	0.38±0.10	0.35±0.04	0.36±0.07

*Mean ± SD for each experimental condition SP and SS are reported. Statistical significant difference is set by *P < 0.05. Expiratory minute ventilation (V Ė), oxygen consumption rate (V Ȯ2), CO2 production rate (V ĊO2), all reported in [l/min], respiratory exchange ratio (RER) [number]. Mean arterial pressure (MAP) [mmHg], heart rate (HR) [bpm], cardiac output (C Ȯ) [l/min], total peripheral resistance (TPR) [mmHg.s/ml].*

Muscular and cerebral NIRS responses

Muscular and cerebral NIRS results are reported at 25%, 50%, 75% and 100% time to exhaustion in Table 10 for thirteen participants. Values are slightly modified during both test; no differences were observed between the two conditions (SP vs. SS) in paired Student's t-test.

Table 10. *STUDY II. NIRS muscular oxymetry and NIRS cerebral oxymetry values at 25%, 50%, 75% and 100% time to exhaustion.*

Nirs muscular oxymetry	Condition	25%	50%	75%	100%
Sat	SS	64.20±9.86	66.75±9.17	68.83±9.23	67.92±9.14
	SP	63.46±8.48	64.68±11.05	65.31±11.93	66.03±11.20
THb	SS	110.67±43.75	113.53±43.02	114.36±38.92	111.30±37.27
	SP	110.01±41.62	108.06±37.56	106.75±33.71	105.93±33.78
O2Hb	SS	68.48±24.24	73.12±23.55	76.29±20.94	73.01±18.22
	SP	68.06±22.13	67.32±18.41	67.03±14.76	67.67±16.55
HHb	SS	42.19±22.24	40.41±21.55	38.07±20.40	38.29±21.02
	SP	41.94±22.88	40.74±23.55	39.72±23.40	38.27±21.68
Nirs cerebral oxymetry					
Sat	SS	52.26±9.82	53.06±9.03	54.06±8.98	54.20±9.48
	SP	54.10±7.26	57.02±9.42	57.02±8.80	57.42±9.80
THb	SS	56.57±13.63	57.57±14.95	57.67±14.34	57.54±14.25
	SP	54.85±11.27	56.71±12.85	57.07±14.66	57.55±15.61
O2Hb	SS	30.33±11.19	31.36±11.61	32.05±11.59	32.14±11.89
	SP	30.37±9.69	33.38±12.21	33.68±13.02	34.23±13.67
HHb	SS	26.25±5.74	26.21±5.70	25.62±4.99	25.40±4.72
	SP	24.48±2.89	23.33±3.02	23.38±3.04	23.32±4.44

*Mean ± SD for each experimental condition SS and SP are reported. Statistical significant difference is set by *P < 0.05. Oxy- (O2Hb), deoxy- (HHb), total Hb (THb) hemoglobin concentrations [μMol/l], (Sat) oxygen saturation in tissue [%].*

Lactate Glucose and Hemoglobin Concentration

Plasma lactate concentrations increased significantly by 10min from resting values of ~ 1 mmol/l (P <0.001) but stabilized thereafter and did not change significantly during exercise or between trials (SS, 5.01 ± 2.29 mmol/l; SP, 4.66 ± 1.85 mmol/l). Plasma glucose concentration decreases significantly from rest (SS, 4.69 ± 0.54 mmol/l; SP, 4.73 ± 0.45 mmol/l) to exercise (SS, 3.97 ± 0.34 mmol/l; SP, 3.95 ± 0.39 mmol/l), in both conditions (P <0.001). Hemoglobin concentration remains stable from rest (SS, 17.34 ± 1.38 g/dl; SP, 17.13 ± 1.35 g/dl) to exercise (SS, 17.47 ± 0.93 g/dl; SP, 17.29 ± 1.37 g/dl) condition and did not change significantly.

3.4 Discussion

This study showed that the administration of SS on healthy non-tobacco users did not change, compared to SP, the perception of fatigue in endurance exercise until exhaustion. A non-significant trend to increase in time to exhaustion (+18.6%) was observed after snus administration compared to placebo, similarly to a significant increase (+17%) described after nicotine patch administration (Mundel and Jones 2006). Although pharmacokinetic assessment confirmed nicotine adsorption through snus, no effects were however observed for cardiovascular, respiratory and biochemical parameters compared to placebo.

In our study, we observed a non-significant increase in time to exhaustion whereas Mundel and Jones found a significant effect. Interestingly, in both studies the magnitude in time to exhaustion increase was the same (18.6% vs. 17%). In our sample, three participants under snus condition showed an increase (53,7%) in total mean time to exhaustion. We recruited 14 participants from different sports, so that could be that the different training background may be a possible factor for inter-individual variability in time to exhaustion. If it assumed that time to exhaustion may be determined by the depletion of energy substrates such as muscle glycogen, then individual's ability to use glycogen may be a relevant factor when exercise is at 70-80% of the maximum capacity aerobics (Saltin and Karlsson 1971). Noteworthy, we tried to control for this potential factor by asking to participants to maintain the same dietary conditions between the two sessions (SS and SP), i.e. to record their diet in the 24 h period before the EXP2, and to follow the same diet before each subsequent experiment.

In literature there are only few studies that showed nicotine effect whereas for caffeine there are more studies suggesting an increase of time to exhaustion. Indeed, two recent studies (Marshall 2010; Desbrow et al., 2012) showed that caffeine significantly increased time to exhaustion in comparison to placebo. Authors concluded that, due to the side effects reported at high caffeine doses, it might be more beneficial for athletes to ingest low doses as the adverse effects may surpass the ergogenic benefits.

We observed a non-significant effect of snus on perception of effort as similarly to

Mundel and Jones's study with nicotine patch (2006). Borg's RPE scale is an indicator that integrates both the perception of exertion and signals elicited from the peripheral working muscles and joints. Furthermore, Borg's scale integrates information from the cardiovascular and respiratory functions and from the Central Nervous System (Borg 1982b). Borg's RPE can be considered the more reliable and feasible tool for monitoring exercise intensity. Indeed, in a recent study, Scherr and colleagues (2013) have compared a subjective indicator of exertion (Borg's RPE) to a metabolic indicator (lactate levels) and to a hearth rate (HR) of intensity during exercise. These authors have evaluated 2.560 Caucasian men and women that completed incremental exercise tests on treadmills or cycle ergometers. Data showed that the relationships between RPE and exercise intensity—assessed by blood lactate, and heart rate is strong and independent of age, gender, medical history, level of physical activity and exercise modality. In our study, five participants showed side effects whose symptoms may have masked changes in perception of effort during exercise.

Our results could not be extended to regular snus users (Kobiella et al., 2011). It could be interesting to investigate the effects of nicotine in snus users under abstinence and satiety condition (for instance, similar to Escher et al., 1998, see below). In fact, factors such as tolerance to nicotine effects, and/or changes in nicotinic acethylcholine receptors availability should influence the response to nicotine absorbed through snus.

Another limitation is the lack of a positive standard comparator, i.e., an experimental group under which perception of fatigue decrease and/or time to exhaustion increase. For instance, a double-blind, counterbalanced study by Killen and colleagues (2013) examined the effects of caffeine on session ratings of perceived exertion following 30 min constant-load cycling. Fifteen participants completed two tests following ingestion of 6 mL/kg of caffeine or placebo. Caffeine resulted in a significantly lower RPE for caffeine versus placebo.

An important set of variables account to peripheral sympathetic control of endurance exercise. For this reason, we included in this study cardiovascular, respiratory, biochemical and neurophysiological assessment. No significant effects were seen between the two groups (SS vs. SP) for all these measurements. According to Van Duser and Raven's (1992) our data also showed that anaerobic energy production is higher after nicotine, and that there is an early increase in tachycardia response and in mean arterial pressure at relative submaximal workload. Nicotine is an activator of the sympathetic nervous system. These effects cause release of the neurotransmitter norepinephrine, skeletal muscle tremors, increased blood flow, elevated heart rate and blood pressure (Yoshida et al., 1994). Under our conditions, nicotine effect delivered by snus does not influence peripheral parameters probably because masked by sympathetic activation. The possibility to measure directly oxyhemoglobin concentration and deoxyhemoglobin concentration (HHb) in muscle tissue and in the frontal cortical areas leads us to evidence that cerebral HHb is higher in SS than in SP supporting the hypothesis of a major activity induced by nicotine as a central stimulator.

Other possible CNS effects could influence performance. Escher and colleagues (1998) tested 20 smokeless tobacco-using athletes while both using and after abstaining from smokeless tobacco. The tests were reaction time, maximum voluntary force, and maximum rate of force generation of the knee extensors on dynamometer. The authors showed that smokeless tobacco use has no effect on reaction time but may influence maximum voluntary force and maximum rate of force generation.

In conclusion, since nicotine obtained through snus under our experimental condition could be considered the effect of a maximal obtainable dose, we suggest that snus use in healthy non-smoker participants does not modify perception of effort and does not increase time to exhaustion on exercise endurance.

4. STUDY III.

Effects of snus administration in Iowa Gambling Task in men

4.1 Introduction

Decision-making comprises a complex process of assessing and evaluating short-term and long-term costs and benefits of competing actions. The output of the decision-making process is determined by an interaction between impulsive or emotionally based systems, responding to immediate (potential) rewards as well as losses or threats, and reflective or cognitive control systems controlling long-term perspective (Bechara 2005). Research has shown that areas such as the ventromedial prefrontal cortex (VMPC), amygdala, insula, somatosensory cortex, dorsolateral prefrontal cortex and hippocampus are all involved in various aspects of decision-making (Bechara et al., 1999; Bechara et al., 2000; Bechara et al., 2003; Gupta et al., 2009; Gupta el al., 2011).

An experimental tool to study decision-making is the IGT. This test was designed to simulate real-life decisions in terms of uncertainty of outcomes and variable reward and punishment (Bechara et al., 1994). In this task, participants choose a card from four decks of cards. Each card indicates either gain or loss of money. Participants are instructed to earn as much money as possible. Two of the four decks are "risky", containing cards with bigger gains and losses than the other two decks. The sustained selection of the risky decks ends up in overall loss. Therefore, the choice behaviour becomes "cautious" in healthy participants during the task. However, patients with lesions in the prefrontal cortex and substance abusers persist on the risky decks. The task has been used in studies that had consistently demonstrate that impaired decision-making contributes to loss of control and substance abuse, especially when it requires appraisal of the potential rewards or punishments (Bechara et al., 2000). In literature some studies in substance abusers have assessed their performance on the IGT. Grant et al (2000) administered the task to poly-substance abusers versus subjects not using illicit drugs of abuse. Other studies have been done using IGT to test the effects of alcohol (Johnson et al., 2008), cocaine (Monterosso et al., 2001; Vadhan et al., 2009), marijuana (Vadhan et al., 2007; Vaidya et al., 2012), opiate-dependent (Pirastu, et al., 2006) and MDMA (Quednow et al., 2006).

Only two studies investigated the relationship between decision-making and nicotine effects. Xiao and colleagues (2008) administered the IGT to 10th-grade Chinese adolescent smokers. Nine out of 10 of teenagers who said they had smoked within the previous seven days did more poorly on the IGT. Another study (Mitchell, 2004) demonstrated an increase in impulsive decision-making when smokers were deprived of nicotine but the researcher did not used the IGT. To our knowledge, no study has been done nicotine administered as a pure substance or through other nicotine-contained smokeless products by using the IGT. One of the popular STP consumed orally, not smoked, traditionally produced and used in Sweden is snus (Rutqvist et al., 2011). It is typically placed between the upper lip and the gum for approximately 30 min before it is discarded. Sweden is the only country in the EU granted special exemption to manufacture and snus sales (Anon 1992; Ahlbom et al., 2007; Fagerström and Schildt 2003*).* In 2006, snus was introduced in USA and Alpert and colleagues (2008) reported an increasing trend in its use.

Therefore, the aim of this study was to measure the effect of snus administration in IGT in men.

4.2 Material and methods

4.2.1 Participants

We recruited 40 healthy men volunteers (18 – 65 years) non-smokers and non-snus users. All participants were recruited by local advertisements in the metropolitan area of Verona (Italy) and in the University of Verona.

4.2.2 General Design

At EXP1 demographics of subject's information were collected. All participants received the information letter to deliver to their family doctor. They were informed about the procedures and risks, and they signed an informed consent form before to start the experiment.

The study was a double-blind crossover design study comparing the effect of Swedish Snus (SS) vs. Swedish Snus Placebo (SP) on IGT (Figure 11). Before and after IGT, subjects were administered with the Profile of Mood of State (POMS) questionnaire. Subjects were randomized to blindly receive SS or SP on EXP1 day and on EXP2 day in accordance with a random sequence generator schedule generated by software online (www.random.org), prior to the start of the study. Session duration was approximately 75 min on EXP1 and 60 min on EXP2. The study was approved by the institutional ethical committees of local National Healthcare System Unit (ULSS20, Verona, Italy) and conformed to the 1964 Declaration of Helsinki.

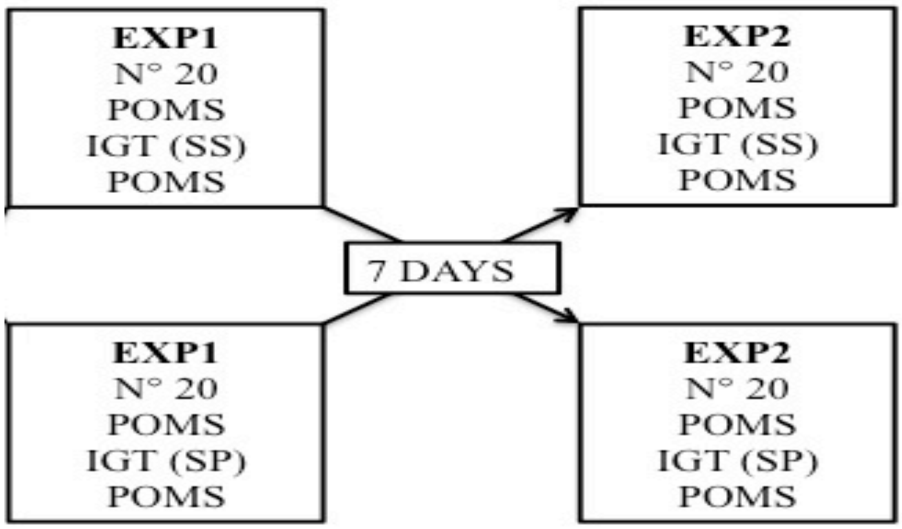

Figure 11. STUDY III. Schematic representation of experimental design. Abbreviations: EXP1: Experiment 1; EXP2: Experiment 2; POMS: Profile of Mood State questionnaire; IGT: Iowa Gambling Task; SS: Swedish Snus; SP: Swedish Snus placebo.

4.2.3 Protocol

The protocol consisted of two experiments.

EXP1. At arrival in the Neuropsychopharmacology lab in the University of Verona the demographics of participants were collected. The measurement of exhaled carbon monoxide (CO) level was performed using the EC50 Micro Smokerlyzer† (Bedfont Scientific Ltd.) and the average was reported in the Case Report Form (CRF). After CO measurement, subjects seated in front of the computer and filled the POMS. IGT was administering by using the same software used in Pirastu et al (2006). We administered SS or SP twenty-five minutes before starting IGT (Figure 12). During these 25 minutes subjects could read magazines, but not interact with technological tools such as mobile phone or computer. At the end of IGT, subjects spitted out the product. Immediately after, subjects were interviewed about adverse events (if yes, these were scored as mild, moderate or serious). At the end, subject filled out again the POMS questionnaire.

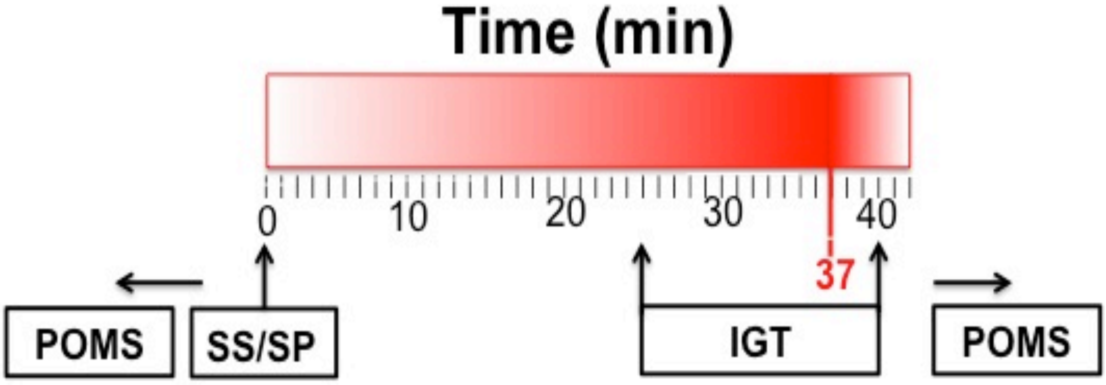

Figure 12. STUDY III. Schematic time plan design. Red bar is the estimated nicotine plasma concentration (the mean of Tmax is 37.1 ± 10.2 min according to Lunell and Curvall 2011). Abbreviations: POMS: Profile of Mood State questionnaire; IGT: Iowa Gambling Task; SS: Swedish Snus; SP: Swedish Snus placebo

EXP2 was performed with the same procedure of EXP1 (without data collection of the demographic of the sample) and it took place at least 7 days later.

4.2.4 Materials

Iowa Gambling Task

The IGT is a computerized version of the gambling task with an automated and computerized method for collecting data. Four decks of cards labelled A, B, C and D were displayed on the computer screen. The backs of the cards all look the same as real decks of cards. The participant started the task with a sum of make-believe money in his or her account ($2,000), represented by a green bar that changes in length as the participant ‘‘wins’’ or ‘‘loses’’ money during the task. The subject was required to select one card at a time from one of the four decks. When the subject selected a card, a message was displayed on the screen indicating the amount of money the subject won or lost. The computer controlled the pre-programmed schedules of gain and loss. Turning each card gave an immediate reward of $100 in Decks A and B and $50 in Decks C and D (Figure 12). As the game progressed, there were also unpredictable losses among the card selection. Total losses amounted to $1,250 in every 10 cards in Decks A and B compared to $250 in Decks C and D. Decks A and B were equivalent in terms of overall net loss, and Decks C and D were equivalent in terms of overall net gain over the course of the trials. The difference was that in Decks A and C, the punishment was more frequent but of smaller magnitude, whereas in Decks B and D, the punishment was less frequent but of higher magnitude. Thus, Decks A and B were disadvantageous because they yielded high immediate gain but a greater loss in the long run (i.e., net loss of $250 for every 10 cards), and Decks C and D were advantageous in that they yielded lower immediate gain but a smaller loss in the long run (i.e., net gain of $250 for every 10 cards). In this study, an overall net score of the IGT was calculated by subtracting the total number of selections from disadvantageous decks (A and B) from total number selections from advantageous decks (C and D). We divided all 100 cards in five block cards called Time 1 (T1; from 1 to 21 card), Time 2 (T2; from 21 to 40 card), Time 3 (T3; from 41 to 60 card), Time 4 (T3; from 61 to 80) and Time 5 (T5; from 81 to 100) respectively and we calculated the net scores values ((C + D) – (A + B)) for each blocks cards.

Figure 12. STUDY III. Deck contingencies on the Iowa gambling task. Image taken from Buelow et al (2013).

Snus and Placebo

Snus (according to the GothiaTek standard) is a low-nitrosamine, moist oral tobacco product with water content of approximately 45%–55% and a pH of approximately 8.5. Lunell and Lunell (2005) describes the nicotine delivery and uptake from snus sachets. The mean time to maximum nicotine plasma concentration (Tmax) is 37.1 ± 10.2 (SD) min (range: 24–60 min) for 1 g Swedish portion snus (Lunell and Curvall 2011). Fagerstrom et al (2012) show a lack of serious AEs that require medical intervention. The placebo (SP) was almost identical to the snus in physical appearance, mouth feel, pH, flavouring, and other sensory characteristics, but it did not contain tobacco or nicotine. The placebo is composed of water, oat and cocoa fibers, humectant (E422), flavor enhancer (sodium chloride), acidity regulator (E504, E500) and aromas. In this study we administrated a commercial Catch White Eucalyptus Portion Snus (Swedish Match) 1.0 g - nicotine: 8 mg/portion - for SS and Onico Pepparmint (Swedish Match) 1.0 g for SP respectively.

POMS

The POMS has been used extensively for the assessment of mood in the sport and exercise environments. This questionnaire has 58 items and the factor structure representing six dimensions of the mood construct: Tension, Depression, Anger, Vigor, Fatigue and Confusion. Items were answered on a 5-point Likert scale (0 = not at all, 1 = a little, 2 = moderately, 3 = quite a bit, 4 = extremely). Raw scores were transformed following the standard point table (McNair et al., 1991).

Smoking status

The measurement of exhaled CO concentration, a non-invasive method of assessing smoking status, was measured using the EC50 Smokerlyser (Bedfont Instruments; Kent, UK). The Smokerlyser measures breath CO levels in parts per million (ppm) based on the conversion of CO to carbon dioxide (CO_2) over a catalytically active electrode (Middleton 2000). Exhaled CO concentrations are reported to correlate closely with blood carboxyhaemoglobin concentration in smokers and in non-smokers.

Data analysis

POMS and IGT data (money win, number of choices from advantageous and disadvantageous decks, overall and T1, T2, T3, T4 and T5 net score) were analyzed using paired Student's t-test (SS vs. SP). The normality distributions were tested using the Shapiro-Wilk normality test. For the data that did not have a normally distributions a non-parametric Wilcoxon signed-rank test was used. Then, to compare the condition post with the condition pre and post one sample t-test was used with an alternative hypothesis not equal to 100. Statistical significance will be accepted at $P < 0.05$. ANOVA.

4.3 Results

Participants

Forty non-smoker and non-snus user male subjects were recruited. Means (± SD) age was 23.5 ± 4.6. All subjects completed the study. The level of CO (ppm) was 1.0 ± 0.8 before to start SS test and 1.0 ± 0.9 (means ± SD) before to start SP test. Values defined the subjects as non-smokers, in accordance to Deveci et al (2004).

Adverse events

Twenty-three subjects reported adverse events at the end of SS session and none reported effects adverse at the end of the SP session. Four subjects showed serious side effects as a nausea, dizziness, increase body temperature, anxiety and one of them reported hands tremors (Table 11).

Table 11. *STUDY III. Adverse effects reported by the participants at the end of the SS session.*

Subject Code	Mild	Moderate	Serious
RM01		Sore throat	
AF02	Confuse, Dizziness		
GM04	Dizziness		
PG06	Dizziness		
DN07		Dizziness, Sore throat	
FG08	Nausea	Dizziness, Weariness	
MA09	Tachycardia		
FM13		Stomach-ache, Tachycardia	Nausea, Dizziness, Increase body temperature
PG14	Increase body temperature, Dizziness		
MF15	Dizziness	Empty sensation	
RD17	Nausea, Dizziness		
AA19	Sleepiness	Nausea, Dizziness	
AS20	Dizziness, Hands tremor		
VC22	Nausea, Hands tremor	Dizziness, Sweating	
GM26	Dizziness		
PG29		Dizziness, Tiredness	Nausea
MF30	Nausea, Dizziness, Sweating		
BF33	Nausea, Confuse	Dizziness, Tachycardia	
PF34		Nausea, Dizziness,	

		Increase body temperature	
FF35		Tachycardia	Dizziness, Nausea, Increase body temperature, Hands Tremor
FB36	Dizziness		
BD37		Dizziness, Nausea, Shiver	
MD40		Increase body temperature, Confuse	Nausea, Dizziness

POMS

The POMS questionnaire was measured pre and post for all experiments. Table 12 shows significant differences for Vigor and Confusion factors at pre vs. post administration for SS session; significant differences were observed for Tension, Depression, Anger and Confusion in pre vs. post administration for SP session in the six factors in a paired Student's t-test.

Table 12. *STUDY III. POMS scores (mean ± SD) under SS and SP condition***.**

	SS			SP		
POMS factors	pre	post	p value	pre	post	p value
Tension	41.7 ± 4.1	43.8 ± 9.5	0.6009	42.2 ± 4.5	41.2 ± 5.2	0.0414*
Depression	45.5 ± 5.4	45.8 ± 8.1	0.7947	44.6 ± 5.4	43.2 ± 4.1	0.0001***
Anger	45.1 ± 6.5	44.1 ± 6.3	0.1769	45.3 ± 6.7	43.7 ± 4.5	0.0059**
Vigor	56.8 ± 8.6	51.6 ± 11.7	0.0022**	55.9 ± 9.6	55.7 ± 9.6	0.8048
Fatigue	45.4 ± 6.8	48.0 ± 10.6	0.1255	46.1 ± 9.4	44.3 ± 8.9	0.1010
Confusion	46.6 ± 5.9	49.6 ± 7.2	0.0049**	47.2 ± 7.5	45.7 ± 7.6	0.0281*

Pre: pre IGT; post: post IGT. *p < 0.05, **p < 0.005, ***p < 0.0001, paired Student's t-test comparison between pre exercise and post exercise in each experiment for six factors.

Iowa Gambling Task

Iowa Gambling Task data are reported in Table 13. For all participants, the average money won was (mean ± SEM) $ 3213 ± 140.9 during the SS session and $ 3140 ± 136.3 during the SP session, not significantly different (p = 0.6542; paired Student's t-test). No differences were observed between number of choices from advantageous decks, number of choices from disadvantageous decks and overall net score (SP vs. SS) (paired Student's t-test). Interestingly, a significant difference was observed for net scores in T1 between the SS and the SP condition (p = 0.0499). In the T2, T3, T4, and T5 net scores values in the card blocks no significant differences were observed (see in Figure 13). The two-way ANOVA (time – SS/SP) showed a non-significant interaction between time and SS/SP, F (4, 195) = 2.206, p = 0.0698. There was no main effect of SS/SP, F (1, 195) = 0.3356, p = 0.5630; we did not found a significant different in time, F (4, 195) = 1.132, p = 3427. The randomization factor (SS/SP and EXP1/EXP2), analyzed for a possible form of IGT's learning between EXP1 and EXP2, did not change the significances. Post hoc analysis revealed the two conditions were no significantly different.

Table 13. *STUDY II. Iowa Gambling Task scores (mean ± SEM) under SS and SP condition.*

	SS	SP	p value
Money win ($)	3213 ± 140.9	3140 ± 136.3	0.6542
Number of choices from advantageous decks	68.6 ± 2.5	69.4 ± 2.6	0.7163
Number of choices from disadvantageous decks	31.5 ± 2.5	30.6 ± 2.5	0.7163
Overall net score.	37.1 ± 5	38.8 ± 5.1	0.7163
T1 net scores	4.5 ± 1	7.0 ± 1.1	0.0499*
T2 net scores	8.2 ± 1.3	6.6 ± 1.2	0.2148
T3 net scores	7.6 ± 1.2	9.1 ± 1.3	0.2645
T4 net scores	7.8 ± 1.2	9.0 ± 1.3	0.5615
T5 net scores	8.9 ± 1.3	7.0 ± 1.2	0.1554

Data are expressed as means ± SEM. *p < 0.05, paired Student's t-test comparison between SS and SP condition.

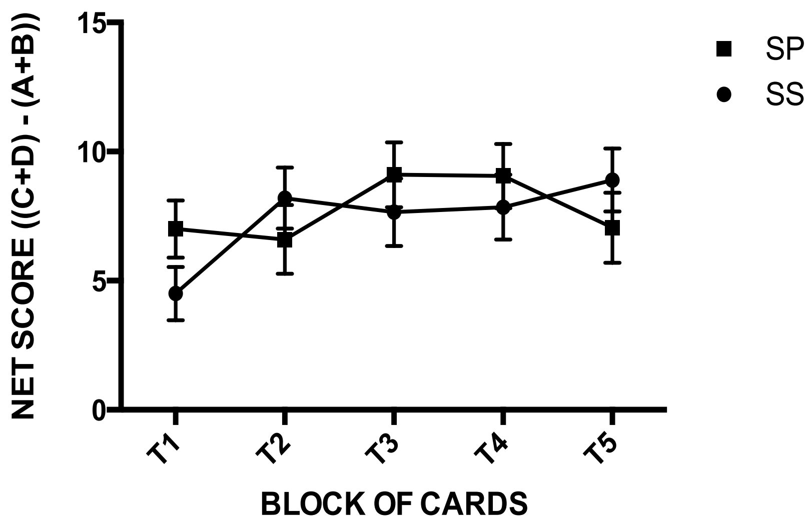

Figure 13. STUDY III. Line graph represents means net scores values (mean ± SEM) for each Time (T1, T2, T3, T4, T5) during SS and SP session.

4.4 Discussion

Nicotine improves cognitive functions such as learning, memory, attention, concentration planning and high-order executive performance (Wesnes and Warburton 1983; Chiamulera 2005). According to these findings Heishman and colleagues (2010) confirmed in their meta-analysis effects of nicotine on fine motor abilities, including attention and memory. Subjects were mainly nonsmokers, therefore avoiding confounding of nicotine withdrawal.

This double-blind randomized study investigated the effects of SS as a source of nicotine on the Decision-Making (DM) process.

IGT, an experimental tool to study DM, provides an initial random choice of decks where the subject is not aware of those which are advantageous and/or disadvantageous. Therefore, as showed in Table 12, both groups showed an initial net score ((C + D) - (A + B)) lower than the final one for each block of cards. In the SP condition, data showed a fluctuating trend for the duration of the test, while in the condition SS there was a steady increase in gain (see Figure 13). According to Goriounova and Mansvelder (2012) the prefrontal cortex, the brain area responsible for executive functions and attention performance is susceptible to the influence of psychoactive substances such as nicotine. Observed nicotine-induced effects on cognitive processes in non-smokers in well-controlled studies may be interpreted as true effects, not confounded by the possible effects of expectancy, nicotine tolerance or withdrawal (Heishman et al., 1998). Furthermore, this increase could be attributed to neurobiological effects of nicotine througth the stimulation of nicotinic receptors at the level of the pre-frontal cortex. Nicotine effects might have allowed the participant to identify the advantageous cards (Xiao et al., 2008).

Subjects under SS condition showed an higher score, but we did not find a significant difference compared to the SP condition. In fact, there were no significant differences between SS and SP for avarage money won. Furthermore, no differences were observed between number of choices from advantageous decks, number of choices from disadvantageous decks and overall net score.

On the other hand, Student t-test showed a significant difference ($p = 0.0499$) in the first block (T1) between the two conditions. A lower net score starting in the SS condition could be due to adverse effects of nicotine. In fact, the subjects under snus condition reported moderate dizziness, sometimes combined with nausea and a perceived increase in body temperature, heart rate and sweating. Dempsey et al (2013) observed that pharmacokinetic and genetic factors underlying individual differences in response to nicotine administration could influence sensitivity in never-smokers.

Only two studies investigated the relationship between decision-making and nicotine effects. Our study was the first study that investigated snus effect in the human cognitive domain. Therefore, further studies are needed to demonstrate if snus could modify the cognitive performance. An interesting phenomenon as appeared in sports environment: smokeless tobacco use in sports (Marclay et al., 2011; Zandonai et al., 2013). Considering the importance of cognition in sport, such an optimization of neurobiological function in our view seems to be beneficial for a variety of sports such as sport games or track and field (Pesta et al., 2013). It would be interesting to study athletes using snus under two conditions (abstinence and satiety) on cognitive performance tasks. Future research should be developed in this direction.

5. STUDY IV

Acute effect of Snus on physical performance and perceived cognitive load on amateur footballers

5.1 Introduction

A recent review by Heishman et al (2010) points to significant effects of nicotine on fine motor abilities and high-order cognitive functions such as attention and memory. Together with changes in cognitive functioning, nicotine administration has been related to significant physiological effects. For instance, Turner and McNicol (1993) observed that nicotine administration during light physical activity was related to increases in heart rate and blood pressure without effects on pulmonary function. In this study, participants were exposed to treadmill exercise at an intensity corresponding to the 60% and 85% of their maximal oxygen uptake (VO_{2max}) under the effects of OST. Tobacco (in comparison to a placebo condition) resulted in significant increases in heart rate, a decrease in stroke volume, and an increase in blood lactate at rest. Furthermore, due to OST-induced increase in plasma nicotine concentrations (boosting anaerobic energy production), a nicotine-induced sympathetic stimulation of the heart was suggested in other research (Van Duser and Raven 1992). Several studies have analysed smoked tobacco effects on heart functions showing that nicotine was able to produce heart rate modulations and decreased heart rate variability (HRV) (Karakaya et al., 2007; Minami et al., 1999). HRV was also used to diagnose different heart diseases in smokers (Barutcu et al., 2005; Pope 2001), and to assess the newborn who had smoker mothers (Franco et al., 2000; Søvik et al., 2001). However, a study by Mundel and Jones (2006) has shown that nicotine (administrated by patch) improved exercise endurance in the absence of peripheral changes (ventilation, heart rate, and blood metabolites) concluding that physical enhancement was attained through a central nervous system mechanism.

One easy and inexpensive way of consuming nicotine is through the use of smokeless tobacco products, like snus. Snus has been traditionally used in Sweden (Rutqvist et al., 2011), sold in small pouches. In Sweden, the increasing trend of snus' use continues (Lund and Lindbak 2007) in all age groups especially among those that were both smokers and snus users (Norberg et al., 2011b). Sweden is the only nation in the European Union (EU) that granted special exemption to manufacture and sell this substance (Anon 1992; Ahlbom et al., 2007) although there is recent evidence of snus consumption in Switzerland (Fischer et al., 2013) and in northern Italy (Zandonai et al., 2013). Snus is of growing popularity in the sport environment due to absence of adverse effects on the respiratory system. Snus use has been associated to high intensity sports in which athletes report lower cigarette consumption than people not engaged in sport practices (Mattila et al., 2013). Some sociological studies showed that the use of snus was more common among team sport athletes than athletes in individual sports (Alaranta et al., 2006; Martinsen and Borgen 2012) and in males than in females (Norberg et al., 2011a; Rolandsson et al., 2014). However, there are no previous studies that have assessed the effects of this product in football players. Moreover, there are no previous studies that have investigated the effect of snus on physical performance, the subjective perceptions of effort, and mental workload in the same experiment.

In the present study, we investigated the effect of snus acute consumption on a group of non-smokers non-snus users amateur football players using a test battery developed to assess different capacities related to physical demands in football (Reilly et al., 2009; Bangsbo et al., 2008). In addition, HRV was used to check the parasympathetic and sympathetic functioning during the experimental session. Crucially, perceived physical effort, subjective felt arousal, and perceived mental workload were also assessed. The reasons why we consider this study pertinent are: 1) there are no studies assessing the effects of snus on physical performance, perceived physical effort, and perceived mental workload in the same experiment; 2) nicotine (the major component of snus) has been included on WADA's Monitoring Program recently; 3) snus is a substance with growing popularity in sport; 4) No previous study has investigated the effect of snus on football players. Therefore, the aim of this study was to assess the effect of snus on physical performance, heart rate variability, and subjective arousal and mental workload in non-smokers non-suns users amateur football players.

5.2 Materials and Methods

5.2.1 Participants

A total of 18 male amateur football players (age: 22.5 ± 1.5 years old; height 1.80 ± 0.056 meters; weight: 75.6 ± 7.37 kilograms; body mass index: 23.30 ± 1.44;

mean ± standard deviation) non-smokers and non-snus users took part in this study. All participants were recruited from the Faculty of Sport Sciences, University of Granada (Spain).

The study was approved by the Ethics Committee of the University of Granada and conformed to the 1964 Declaration of Helsinki.

5.2.2 General Design

The study was a double-blind randomly assigned crossover design, comparing the effect of snus vs. placebo on physical performance -measured using a Fitness test battery-, and perceived effort -measured by the rating of perceived exertion (RPE)-, subjective arousal, and mental workload -measured using visual analogue scales-. The Fitness test battery consisted of 4 tests: Handgrip Dynamometric Test, Counter-movement Jump, 5 x 10 meters Agility test, and Yo-yo intermittent recovery test.

The protocol consisted of two sessions (EXP1 and EXP2) with 5 days of recovery and substance wash-out between each other. A training session was carried out a week before EXP1 to ensure participants' familiarization with the tests. Half of the participants blindly received Swedish Snus (SS) on EXP1 day and Swedish Snus Placebo (SP) on EXP2 day, and the remaining half of the participants received SP on EXP1 day and SS on EXP2 day. Participants were randomly assigned to one of the two counterbalanced conditions. Each experimental session lasted for about 90 minutes. Before and after the Fitness test battery, participant's subjective arousal and mental workload was assessed (Figure 14).

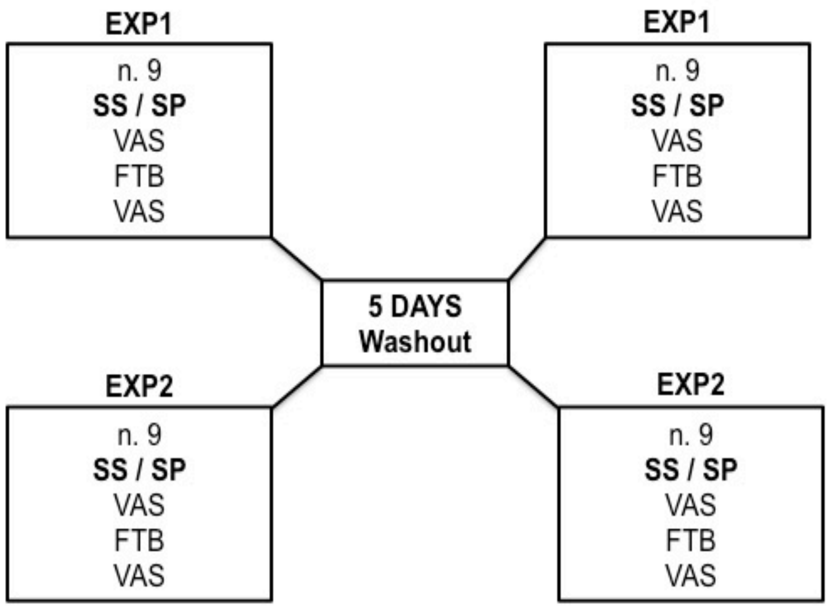

Figure 14. STUDY IV. Protocol and experimental design. Abbreviations: EXP1: Experiment/assessment session 1; EXP2: Experiment/assessment session 2; VAS: visual analogue scale (subjective arousal and mental workload); FTB: Fitness Test Battery; SS: Swedish Snus; SP: Swedish Snus Placebo; n.: number of participants.

5.2.3 Protocol

Familiarization session. Participants arrived at the Faculty of Sport Sciences of the University of Granada, by 08.00 a.m. They were informed about the study, its procedure and risks, and then signed an informed consent before to start the experiment. Then participants performed the Fitness test battery. The experimenter supervised the correct performance of this test.

EXP1. Participants arrived at 08:00 a.m. to the laboratory. Before the start of the experimental session, they were asked the following control questions: “How many hours did you sleep last night?”, and “Have you had any stimulant such as tea or coffee this morning before coming to the lab?”. The use of the visual analogue scales, the perceived readiness (PR) and perceived exertion (RPE) scales was subsequently explained to the participants. Then, measurement of exhaled carbon monoxide (CO) level was performed using the EC50 Micro Smokerlyzer† (Bedfont Scientific Ltd.). After this test, participants laid down for 10 minutes in a supine position to record their basal HRV, which was recorded during the entire session with a Polar RS 800 (see HRV measurement for further details). Subsequently, participants took the sachet of either SS or SP from the box (for further details see

Snus and Placebo in Materials section). They were instructed to place the SS or the placebo substance in the anterior part of the mouth within the upper gingiva (Time zero = T0) and to keep it in their mouth until performance of the Yo-Yo Test. After taking the SS or SP, participants were invited to remain lying on a mat during 35 minutes in a supine position to record their HRV. Thirty-five minutes after intake (T0), psychological parameters (subjective arousal and mental workload) were recorded by means of a visual analogue scale. After 40 minutes from T0, participants stood up and started the Hand Dynamometric Test. Next, after a brief warm-up, participants performed a vertical jump test as indicator of instantaneous power production. The vertical jump test involved two countermovement jumps (Ergojump, Rome, Italy) interspersed by 1 min rest. Only the best jump from each subject was used in data analysis. Next, agility was assessed by means of a 10 x 5m Agility test. After 70 minutes from T0 they spat out the corresponding substances (SS or SP) and started the Yo-Yo Test (Figure 15). At the end of the Yo-Yo Test subjective arousal and mental workload were recorded by means of the visual analogue scale, and immediately after participants were interviewed by researchers about nicotine adverse effects (if yes: mild / moderate/ serious) (Table 13). Global RPE and readiness perception were recorded several times during the Fitness test battery.

EXP2 was performed exactly following the same procedure and timetable of EXP1 for each participant, and took place 5 days later.

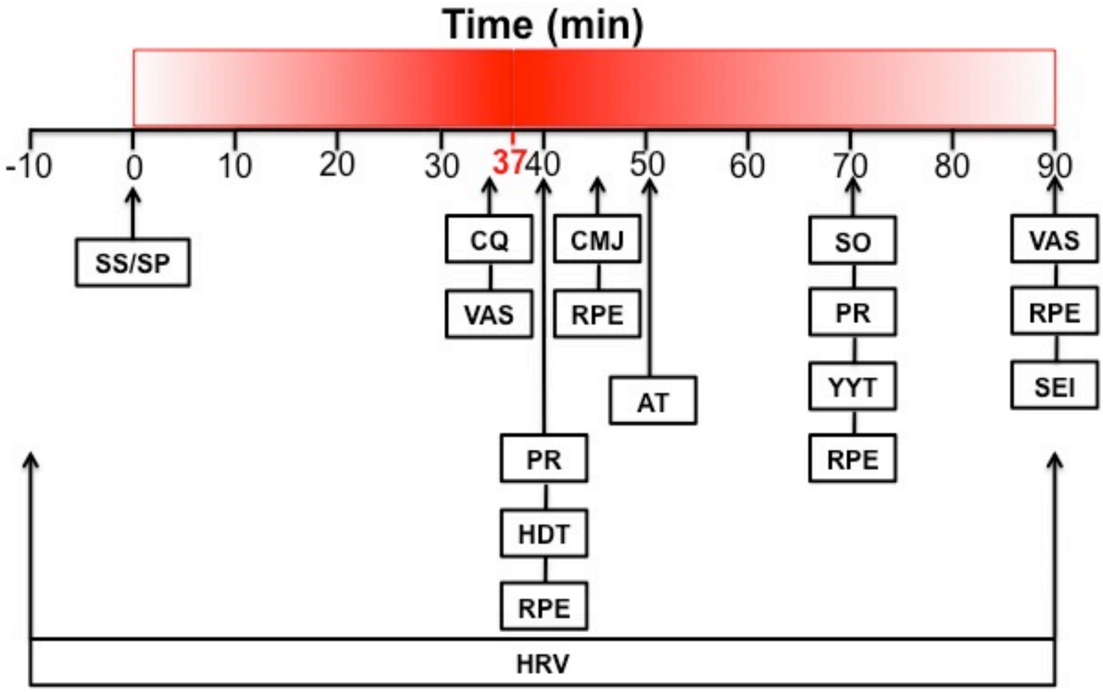

Figure 15. STUDY IV. Schematic time plan design. Red bar is the estimated nicotine plasma concentration (the mean of Tmax is 37.1 ± 10.2 min according to Lunell and Curvall, 2011). Abbreviations: SS: Swedish Snus; SP: Swedish Snus Placebo; RPE: rating perceived exertion (Borg's scales); CQ: control questions; VAS: visual analogic scale (subjective arousal and mental workload); PR: perceived readiness; HDT: hand dynamometric test; CMJ: countermovement jump; AT: agility test; SO: substance (Snus / Placebo) out; YYT: Yo-yo intermittent test; SEI: side effects interview; HRV: heart rate variability.

5.2.4 Materials

Snus and Placebo

Snus is a low-nitrosamine, moist oral tobacco product with water content of approximately 45%–55% and a pH of approximately 8.5. For Snus and Placebo delivery and uptake we followed Lunell and Lunell (2005) methodology. The mean time to maximum nicotine plasma concentration (Tmax) is 37.1±10.2 (SD) min (range: 24–60 min) for 1 g Swedish portion snus (Lunell and Curvall 2011). Fagerstrom et al., (2012) show a lack of serious AEs that require medical intervention. The placebo (SP) was almost identical to the snus in physical appearance, mouth feel, pH, flavouring, and other sensory characteristics, but it did not contain tobacco or nicotine. The placebo is composed of water, oat and cocoa fibers, humectant (E422), flavor enhancer (sodium chloride), acidity regulator (E504, E500) and aromas. In this study, we administrated a commercial Catch White Eucalyptus Portion Snus (Swedish Match) 1.0 g - nicotine: 8 mg/portion - for Snus and Onico Pepparmint (Swedish Match) 1.0 g for Placebo respectively.

Smoking status

The measurement of exhaled CO concentration, a non-invasive method of assessing smoking status, was measured using the EC50 Smokerlyser (Bedfont Instruments; Kent, UK). The Smokerlyser measures breath CO levels in parts per million (ppm) based on the conversion of CO to carbon dioxide (CO2) over a catalytically active electrode (Middleton and Morice 2000). Exhaled CO concentrations are reported to closely correlate with blood carboxyhaemoglobin concentration in smokers and in non-smokers.

Arousal and mental workload

Before Snus or Placebo ingestion and immediately after finishing the Fitness test battery participants were asked to rate their perceived arousal and perceived mental and physical fatigue using a visual analogue scale from 0 (nothing) to 10 (top) in response to the following questions:

1) “What is your activation level now?”

2) “What is your physical fatigue level now?”

3) “What is your mental fatigue level now?”

At the end of the session, participants were asked to use the visual analogue scale again according to the following questions:

1) “Finally, how would you rate the overall mental load for this experimental session?”

2) “How do you feel now, once the session has finished?”

Rating of Perceived Exertion

Rating of perceived exertion was measured with the Borg 15 point scale (RPE 6-20) (Borg et al., 1985) and with a 10-point category-ratio (RPE CR10) (Noble et al., 1983). Participants were given verbal encouragement during the fitness tests. Immediately after the handgrip test, Counter-movement Jump, and Yo-Yo Test RPE were asked.

Perceived readiness

The value for perceived readiness (1-5 scale) (Nurmekiv et al., 2001) was also taken prior to the handgrip and Yo-Yo tests. This scale determines the grade of recovery that subjects perceived from 1-point (“not recovered at all”) to 5-points (“completely recovered”). Before beginning the handgrip and Yo-Yo Test ratings of perceived readiness were asked.

HRV measurement

Recordings were performed using a Polar RS800 HR monitor set to RR interval mode (Polar Electro, Kempele, Finland) together with an electrode transmitter belt (Polar Wearlink Wind, Polar Electro, Kempele, Finland), after application of conductive gel as recommended by the manufacturer. Data were transferred to Polar Pro Trainer 5 software (Polar Electro, Kempele, Finland) and afterwards analysed by means of Kubios HRV Analysis Software 2.1 (The Biomedical Signal and Medical Imaging Analysis Group, Department of Applied Physics, University of Kuopio, Finland).

Handgrip dynamometry

An electronic dynamometer (Takei TKK-5401, Tokio, Japan) was used to determine handgrip strength in both right and left hands. The dynamometer was adjusted for each subject's hand size and the participants were kept in stand position with the arms parallel to the ground and with the elbow joint maintained at 90 degrees of flexion. The participants were instructed to perform a maximal isometric contraction. Each subject was allowed 2 trials non-consecutive per arm, and the highest value was recorded.

Countermovement jump

Muscle power was evaluated using a wireless inertial measurement unit (FreePower®, Sensorize, Rome, Italy), which was validated by Squadrone et al (2012). Two standardized counter-movement jumps separated by 2-min rest interval were performed. The wireless inertial measurement unit of FreePower® was positioned approximately at the centre of body mass, placing the belt around the waist. Participants started from a standing position with hands on their hips and were instructed to perform a fast downward movement up to 90° of knee flexion followed by an upward movement trying to jump as high as possible. Maximum jump height was registered for further analysis.

Agility test (5x10)

Agility was evaluated using a 10 x 5 meters maximal shuttle run on an indoor running track following the protocol of Eurofit (1998). After 1 practice trial a maximum test was performed. Performance time was recorded with an accuracy of 0.01 seconds using Chronometer application for Iphone 4.

Yo-Yo recovery intermittent test

The Yo-Yo test was performed using the level-1 version of the test, following the guidance defined by the test's creator (Bangsbo 1996). The level-1 Yo-Yo test is a progressive shuttle running test that allows 10 seconds of active recovery after every second 20 meters shuttle. Running speeds are dictated by an audible cue played from a CD. Participants must be at one end of a 20 meters base every time a signal is played. Yo-Yo test performance is considered as the total distance covered by the subject when they drop out.

Data analysis

Data are presented as mean values ± SD and were analysed with IBM SPSS 20.0 using paired-sample t-tests to compare differences between the SS and SP ingestions conditions. In order to address any learning/familiarization effect, data from EXP1 and EXP2 were compared using paired-sample t-tests. Bonferroni post-hoc correction for multiple comparisons was applied obtaining a level of significance of $p=0.003$.

For HRV data, the recordings were preprocessed to exclude artifacts by eliminating RR intervals, which differed more than 25% from the previous, and the subsequent RR intervals (Malik et al., 1989). Removed RR intervals were replaced by conventional spline interpolation so that the length of the data did not change (i.e., resulting in the same number of beats). We used the smoothness prior method with a Lambda value of 500 to remove disturbing low frequency baseline trend components (Tarvainen et al., 2002). Regarding linear analysis during the experimental conditions, the mean R-R interval (RRi), root-mean-square difference of successive normal R-R intervals (rMSSD) were calculated for the time domain. Geometric Poincaré Plot index (SD1) was also calculated.

5.3 Results

No significant differences were found between data from EXP1 and EXP2 (all ps>0.003), showing that there were no learning or fatigue carry-over effects across experimental sessions.

Control variables

No significant differences were observed in relation to sleeping hours between Snus (6.94±1.11) and placebo (6.67±1.09) sessions. Regarding Smoking status assessment, the level of CO (ppm) before completion of the fitness battery test was 2.97±1.45 for the snus condition and 3.54±3.83 for the placebo condition respectively. Values defined the participants as non-smokers, in accordance to Deveci et al (2004).

Results for all dependent variables (Subjective arousal, perceived mental workload, Rate Perceived Exertion, Perceived readiness, HRV measurement, Handgrip dynamometry, Countermovement jump, Agility test, Yo-Yo test) for snus and placebo conditions are shown in Table 14.

Regarding HRV measurements, our data showed a significant effect in the snus condition (basal measure before intake vs. after intake) in the RRi, rMSSD and SD1 variables. No significant differences were observed in the placebo condition. No significant differences before substance intake were observed between the SS and SP conditions. Interestingly, there were significant differences for all HRV variables when comparing the snus and placebo conditions after substance intake (Table 15).

Side effects

Twelve participants reported adverse symptoms at the end of the snus condition session and only one reported adverse effects at the end of the placebo condition session (Table 16). Due to these adverse effects some participants could not complete all assessments during the experimental session. This is the reason of the reduced N in some of the statistical comparisons (see Table 15).

Table 14. *STUDY IV. Comparison (Bonferroni corrected α=.003) between the SS and SP conditions for all dependent variables included in this study. (Perceived mental workload, Subjective arousal, Rate Perceived Exertion, Perceived readiness, HRV measurement, Handgrip dynamometry, Countermovement jump, Agility test, Yoyo recovery intermittent test).*

	SS			**SP**			**p**
	mean	SD	SE	mean	SD	SE	
CQ1 Sleeping hours	6.94	1.11	0.26	6.68	1.08	0.26	0.097
Smokerlyzer (ppm)	2.97	1.45	0.34	3.53	3.82	0.90	0.563
VAS1 after intake, before FTB (0-10)	4.83	1.85	0.44	5.11	1.74	0.41	0.523
VAS2 after intake, before FTB (0-10)	4.00	2.22	0.52	3.89	2.03	0.48	0.734
VAS3 after intake, before FTB (0-10)	4.17	2.38	0.56	2.94	1.89	0.45	0.005
Perceived readiness before Handgrip test (1-5)	3.71	0.82	0.22	4.43	0.51	0.14	0.001
Handgrip right (Kg)	42.22	5.63	1.33	42.06	4.46	1.05	0.834
Handgrip left (Kg)	37.22	6.28	1.48	38.33	4.73	1.11	0.068
RPE CR10 after Handgrip test	5.72	2.24	0.53	5.67	2.14	0.51	0.842
RPE 6-20 after Handgrip test	14.89	2.85	0.67	14.44	2.62	0.62	0.177
Maximum Jump height (m)	0.43	0.05	0.01	0.44	0.05	0.01	0.634
RPE CR10 after CMJ	6.06	1.87	0.46	5.94	2.11	0.51	0.707
RPE 6-20 after CMJ	14.59	2.55	0.62	14.71	2.14	0.52	0.743
Agility Test (seconds)	18.82	0.81	0.20	18.47	0.62	0.15	0.009
Perceived readiness before YYT (1-5)*	3.60	0.84	0.27	4.00	0.67	0.21	0.037
Distance YYT (m)*	1397.14	447.14	119.50	1.407.86	481.92	128.79	0.926

VO2max YYT (ml*kg*min) *	48.28	3.75	10.03	48.23	4.05	10.81	0.952
RPE CR10 after YYT*	9.41	1.12	0.29	9.20	1.61	0.42	0.556
RPE 6-20 after YYT *	18.67	0.98	0.25	18.53	1.51	0.39	0.685
Mental load after session (0-10)	6.37	2.16	0.54	5.44	1.82	0.46	0.001
VAS1 after session (0-10)	7.19	1.97	0.50	7.37	1.31	0.33	0.628
VAS2 level after session (0-10)	7.87	1.71	0.43	8.00	1.46	0.36	0.783
VAS3 level after session (0-10)	7.12	1.71	0.43	6.44	1.37	0.34	0.102
RPE CR10 after session	7.87	1.85	0.48	7.60	0.99	0.25	0.556
RPE 6-20 after session	16.67	2.38	0.61	16.60	0.91	0.23	0.927

Abbreviations: SD: Standard deviation; SE: Standard error; VAS: Visual Analogue Scale -subjective arousal and mental workload-; VAS1: Activation level; VAS2: Physical fatigue level; VAS3: Mental fatigue level; FTB: Fitness Tests Battery CMJ: Countermovement jump; YYT: Yoyo recovery intermittent test; $VO2_{max}$: maximal oxygen uptake; n = 18; * n=14.

Table 15. *STUDY IV. HRV measurements.*

	SS			**SP**		
	RRi	*r*MSSD	SD1	RRi	*r*MSSD	SD1
Basal	948.85 ± 119.72*	62.09 ± 28.30*	43.99 ± 20.06*	895.41 ± 131.74	62.76 ± 23.36	44.33 ± 16.59
Activity	665.19 ± 98.21‡	50.11 ± 28.29‡	35.22 ± 19.45‡	887.28 ± 137.54	72.32 ± 30.56	51.23 ± 21.66

* *p*<0.05 Basal vs. Activity condition; ‡ *p*<0.05 Snus (SS) vs. Placebo (SP) condition.

Table 16. *STUDY IV. Side effects described by the participants that reported them at the end of the their SS session.*

Subject Code	Mild	Moderate	Serious
01AAJ		Tachycardia	
02CCJ			Confuse, Dizziness
03CGJ			Nausea, Dizziness
04CBI	Dizziness	Stomach-ache	Empty sensation, Tiredness, Confuse
05EGA	Tiredness		
07LMJ	Dizziness		
09RLF	Sore throat		
11SSS		Nausea, Dizziness	
12BNV	Tiredness	Dizziness	
13HJE	Dizziness		Nausea
16PGM	Dizziness	Sweating, Increase body temperature	Tremor
17MA		Dizziness, Sweating	Empty sensation, Nausea

5.4 Discussion

Nicotine is the main psychoactive substance present in tobacco, targeting neuronal nicotinic acetylcholine receptors (Ortells and Arias 2010). This psychoactive substance has been shown to increase muscle blood flow (Weber et al., 1989), heart rate, blood pressure, level of circulating catecholamine, and vasoconstriction during light exercise (Walker et al., 1999; Cryer et al., 1976). In the present study we investigated the effect of snus (a current common form of tobacco consumption) on physical and mental workload on a sample of amateur sportsmen (football players).

Results from the present study showed that amateur footballers heart rate variability, subjective arousal and mental workload perceptions, and fitness performance were impaired by acute snus intake. Crucially, participants' perceived readiness before some fitness tests, and total mental load after the experimental session, were also boosted as a consequence of snus administration. In addition, 4 of 18 participants (22%) could not complete the fitness test battery due to dizziness, tachycardia, cold sweats and other SS side effects. Regarding participants' HRV, the results showed a decrease after SS administration even before the beginning of the fitness test battery. The results appear to confirm that nicotine leads to reduce vagal tone (Karakaya et al., 2007) as indexed by the rMSSD parameter. The effect of SS was also observed on all the HRV variables analysed compared to the placebo condition.

Our study goes beyond previous research that has investigated the effect of nicotine on HRV (Barutcu et al., 2005; Franco et al., 2000; Karakaya et al., 2007; Minami et al., 1999; Pope 2001). Indeed, these studies used smoked tobacco, and, consequently, they did not control for possible respiratory disorders (Kharitonov et al., 1995), which could have influenced the observed HRV patterns (Hayano et al., 1994). On the contrary, in our study, we were able to avoid possible respiratory disruption focusing on vagal alteration produced by nicotine by administering the product orally.

As already noted, significant beneficial effects of nicotine have been observed on motor abilities and other high-order cognitive functions (see Heishman et al., 2010, for a review) suggesting relevant performance enhancement (Pesta et al., 2013). In particular, significant positive effects of nicotine have been shown on at least six cognitive domains: fine motor coordination, alerting and spatial attention, short-term episodic memory, and working memory (Heishman el al., 2010). Most published studies support that nicotine causes cognitive enhancement in smokers but the influence of nicotine on human performance in non-smokers is less clear. While some studies have failed to detect nicotine-induced cognitive enhancement (Foulds et al., 1996; McClernon et al., 2003) others have even found impairment effects (Heishman et al., 1993).

In our study, participants showed reduced perceived readiness before the Handgrip dynamometry test in the SS condition compared to the SP condition. In the same way, 35 minutes after SS intake, larger mental fatigue values were reported than in after SP intake. Furthermore, once the Yo-Yo Test finished (at the end of the experimental session) participants reported higher mental workload in the SS condition than in the SP condition. Therefore, snus had a negative effect on perceived mental workload, even before starting the physical tests. Perceived arousal was also greater in the SS condition than in the SP condition. Regarding this latter result, research suggests that the effects of nicotine on subjective arousal are dose dependent (Perkins and Stitzer 1998) with mild positive effects at low doses, moderate positive effects at intermediate doses, and negative effects at high doses (Kalman and Smith 2005). Therefore, it is possible that the nicotine intake level achieved in our study was pharmacologically equivalent to a high dose, considering the lack of tolerance in our nicotine-naïve participants. This explanation could be extended to the perceived readiness and perceived mental workload results, explaining the apparent inconsistent results from studies with non-smokers mentioned above. In contrast to the subjective ratings of mental workload and arousal, ratings of Perceived Exertion (RPE) did not differ between the SS and SP conditions. This is consistent with previous studies (Mundel and Jones 2006) that analyzed the effects of nicotine patch on healthy non-smokers and did not observed significant modifications in RPE compared to placebo. Taken together, our results point to a negative effect of snus on subjective evaluation of cognitive load and arousal.

The difference in performance in the Agility test between the two experimental conditions (SS vs. SP) is an interesting result. We suggest that participants performed better under the effects of placebo due to the absence of nicotine side effects on the vestibular system. In fact, Zingler et al (2007) observed that nicotine in non-smokers causes dose-dependent adverse perceptual, ocular motor, vegetative, and postural imbalance effects. These effects can be related to a pharmacological stimulation of the vestibular circuitry. Moreover, given the negative effect of SS on subjectively perceived mental load and arousal, and the high demands of fast responding of the Agility test, one might wonder whether SS intake resulted in lower reaction times to the start of the test. Note that this is highly speculative since we did not collect participants' reaction times, and further research is needed to address this issue.

In contrast to the abovementioned results, no differences between the SS and SP conditions were found for Handgrip dynamometry test, Counter-movement jump, and Yo-Yo test. Furthermore, we found no significant variations under the two experimental conditions (SS vs. SP) for most of the tests in the Fitness test battery which contrast with previous literature that have observed the effect of other psychoactive substance (such as caffeine) when using the same tests (Gant et al., 2010; Mohr et al., 2011). For instance, Gant et al (2010) showed that caffeine administration improved counter-movement jump performance. Moreover, Mohr and colleagues (2011) reported that high-intensity intermittent exercise performance Yo-Yo test was significantly improved by oral caffeine administration in comparison with placebo.

Nicotine absorption varies greatly among individuals. However, according to Lunell and Lunell (2005) the variation in nicotine extraction rates among different snus users could range from 50 to 300 percent in a same snus user since the amount of extracted nicotine could be partially an effect of the intensity with which that portion is manipulated in the mouth. In addition, nicotine side effects may differ between non-smokers and smokers due to tolerance and neuroadaptive processes during prolonged nicotine use (Kobiella et al., 2010). Our study with non-smokers and non-snus users, showed that twelve participants reported after SS administration adverse events such as increased heart rate, dizziness, and nausea (Table 16). Possible explanations could be found in the amount of nicotine absorbed and excessive sensibility to nicotine. It is likely that, as non-smokers, participants were not tolerant to nicotine unpleasant effects. In fact, four participants had to suspend their participation in the fitness test battery before starting the Yo-Yo Test due to serious sides effects (see Table 16, participants 2, 3, 4, and, 13). It is important to note that, compared to rest, exercise can lead to increase in plasma nicotine levels and toxicity due to increased drug absorption during physical exercise (Lenz et al., 1999).

Apart from the side effects reported above, the effect of nicotine varies as a function of nicotine habituation. It would be therefore interesting for future research to investigate the effects of nicotine on smokers athletes and/or snus users, comparing objective and subjective physical and mental performance under conditions of satiety and abstinence. Indeed, SS effects could have been different on non-smokers participants after a familiarization process with this substance although an unethical risk of addiction could appear, which is not in line with the "fair play" and the education of the ideal "athlete 2.0" that rejects the improvement of performance despite the possible health damage (Zabala and Atkinson 2012).

In snus there are presents in addition to tobacco and water, various other ingredients as sodium chloride, sodium carbonate, humectants, and flavoring agents (Rutqvist et al., 2011). One of the limits of this study it is that we did not analyzed nicotine plasma levels concentration to exclude the effect of other substances.

In sum, the results of the present study revealed that amateur footballers' heart rate variability, felt arousal, and mental workload perceptions, and performance in an agility physical test were impaired by acute SS intake. Therefore, taken together, these data argue against snus as an efficient ergogenic aid.

6. CONCLUSIONS

In this research project there were some limitations.

In STUDY I the sample size recruited to compare the relationships between snus and smoking was limited. Studies would be performed based on much larger population.

In the experimental studies (STUDY II, III and IV) the aim was to investigate whether snus improves performance in subjects with no prior experience of snus. We gave them no opportunity to trial the snus and become familiar with it. Therefore when they were given snus in these studies they would have suffered the aversive effects, which could have limited their tolerance. They would practice exercising with snus on many occasions and would develop tolerance to its aversive effects.

Under our experimental conditions in non-smokers non-snus users healthy male subjects did not improve psycho-physiological and cognitive performance. A multidisciplinary experimental approach to investigate the effects of nicotine by snus on smoker athletes and/or snus users, during exercise and cognitive performance under satiety and abstinence conditions is needed. Further researches should be designed on new models mimicking field conditions to producing evidence-based data.

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