Immune checkpoint inhibitors promote T-cell activation, which potentially leads to an increased risk of atherosclerotic events

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Samenvatting
Immuun checkpoint eiwitten zijn betrokken bij het reguleren van T-cel activatie, T-cel overleving en T-cel deling en daarmee een immuunrespons. In de omgeving van de tumor is een langdurige aanwezigheid van antigenen wat tot oververmoeide T-cellen leidt. Hierdoor gaan de T-cellen in deze omgeving meer immuun checkpoint eiwitten tot expressie brengen die activatie van de cellen voorkomen. Bovendien brengen tumorcellen een ligand, nodig voor de werking van immuun checkpoint eiwitten, tot expressie. Beide mechanismen voorkomen dat de T-cel geactiveerd kan worden, waardoor er geen immuunrespons op de antigenen van de tumorcellen komt en de tumor kan ontsnappen aan een aanval van het immuunsysteem. Immuun checkpoint inhibitor (ICI) behandeling is een krachtige kankertherapie. Deze inhibitoren zijn antistoffen die ervoor zorgen dat de rem van het immuunsysteem wordt gehaald, met T-cel activatie tot gevolg. Dit veroorzaakt apoptose van tumorcellen met een sterk verbeterde overleving van oncologische patiënten. ICI-behandeling is in 2011 als eerste goedgekeurd voor een vorm van huidkanker die melanoom wordt genoemd. Momenteel komt naar schatting 50% van de kankerpatiënten in aanmerking voor een vorm ICI-therapie, eventueel gecombineerd met andere anti-kanker therapieën. Doordat ICI-therapie aangrijpt op het immuunsysteem, kan het potentieel ook leiden tot verschillende immuun-gerelateerde bijwerkingen, ook wel toxiciteit genoemd. Toxiciteit wordt gezien in 10-20% van de patiënten die ICI krijgen, en kan variëren van asymptomatisch tot dodelijk. De meest geziene bijwerkingen zijn huidirritatie en ontsteking van de huid, schildklier, darmen en nieren, maar in theorie kan elk orgaan aangedaan zijn. Over het algemeen zijn deze bijwerkingen goed te behandelen met immunosuppressiva, zoals prednison, welke het immuunsysteem remmen. Vanuit eerdere pre-klinische studies, voornamelijk in muizen, is er veel kennis over het effect van checkpoint eiwitten op atherosclerose. Er zijn na de ontwikkeling van ICI voor anti-kanker behandeling dierstudies uitgevoerd die laten zien dat remming van deze eiwitten zorgt voor meer atherosclerose. Bovendien is er ook een toename in het aantal klinische studies dat laat zien dat er in de groep ICI behandelde mensen meer arteriële trombo-embolische events zijn dan de niet ICI behandelde mensen. De drie arteriële trombo-embolische events zijn een hartinfarct, herseninfarct en dood door een hart/vaat probleem. Deze ontstaan doordat de atherosclerotische plaque het volledige bloedvat afsluit en er geen zuurstof meer naar het weefsel achter de blokkade kan. Hierdoor krijgt het hart of de hersenen niet meer genoeg zuurstof en ontstaat er hartschade of hersenschade. Na het vinden van literatuur waarin de relatie tussen ICI gebruik en atherosclerose duidelijk is geworden, is er een systematische review van studies gedaan die ICI-gebruik vergeleken met niet-ICI gebruik en beschreven hoe vaak in beide groepen een hart aanval, herseninfarct en dood door een hart/vaat probleem was voorgekomen. Er waren sinds 2020 maar drie studies die hier iets over hadden beschreven. Ondanks het beperkte resultaat van deze systematische zoekopdracht, wordt het probleem in de toekomst wellicht groter dan we denken. Er is een toename in het aantal mensen die ICIs voorgeschreven krijgen, een verbeterde overleving van de ICI behandelde patiënten en ICI wordt steeds vaker voorgeschreven om de terugkomst van tumorcellen te voorkomen. Hierdoor zal er in de toekomst waarschijnlijk meer atherosclerose en daarmee trombo-embolische events zijn ten gevolge van ICI-behandeling.

Abstract
Immune checkpoint inhibitors (ICIs) have become a powerful cancer treatment. ICIs block co-inhibitory molecules of T-cell activation such as cytotoxic-T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein-1 (PD-1), or programmed cell death protein ligand-1 (PD-L1) resulting in an increased tumor apoptosis. Despite the clinical benefits of ICI use for patients, overactivation of the immune system probably leads to short-term immune-related adverse events (irAEs). The long-term irAEs still need to be clarified. However, preclinical research shows ICI use is related to atherosclerosis. Obstructed vessels, by the plaque or the thrombus on top of the plaque, can result in thromboembolic events and are more observed in patients who use ICIs in clinical trials. After review of the literature, we performed a systematic review of phase II and III studies which compared ICI use with non-ICI use. Three articles described the number of arterial thromboembolic events and were included, however, there was no difference in the number of atherosclerotic events observed.

Introduction
Immunotherapy has grown into an important cancer treatment. It increases the activity of the immune system, resulting in increased antitumor immunity. There are different types of immune therapy, which are: immune checkpoint inhibitors (ICIs), cytokines, chimeric antigen receptor T-cell (CAR-T-cell), monoclonal antibodies, and vaccines[1]. Immunotherapy results in increased overall survival of cancer patients[2]. ICIs are monoclonal antibodies that block co-inhibitory molecules such as cytotoxic-T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein-1 (PD-1), or programmed cell death protein ligand-1 (PD-L1). CTLA-4, PD-1, and its ligand inhibit the (initially) activated T-cells during immune response [3]. Immune-related adverse events (irAE) are a common side effect of ICIs and may occur in any organ. Most frequent, irAE include hepatitis, pneumonitis, colitis, or thyroiditis. The underlying pathophysiology is not fully understood. Cardiovascular irAE is rare, 1-1.5%, and mainly includes myocarditis[4]. The association between ICI use and atherosclerosis has raised concerns since the immune system's role in atherosclerosis is confirmed. Preclinical and clinical evidence shows that respectively atherosclerosis and arterial events are more reported after treatment with ICIs, however, the association between them is not extensively investigated and documented yet [5,6]. This review will describe how treatment with PD(L)-1 and CTLA-4 works and how it can lead to the development or progression of atherosclerotic plaques in oncological patients. Moreover, it will systematically assess the risk of major atherosclerotic events in ICI-using patients.

Role of checkpoint proteins in T-cell activation
Activation of T-cells leads to survival and proliferation of T-cells[1]. Two signals are needed for the activation of naïve T-cells (Figure 1). The first incomplete signal is the binding of the T-cell receptor (TCR) to the antigen presented on the major histocompatibility (MHC) molecule of the antigen-presenting cell (APC) such as the dendritic cell and the macrophage. The second signal is one of the co-stimulatory signals, such as Cluster of Differentiation 28 (CD28) binding to their ligand B7.1 and B7.2[1,7]. This second signal amplifies the first signal and leads to complete activation of the T-cell[7]. Next to the ligation of a receptor to a ligand it is also possible that pro-inflammatory cytokines function as a co-stimulation[1]. Depending on the type of APC that presents the antigen and co-stimulatory signal, the T-cell will differentiate into a cytotoxic T-cell, T-helper-1 (Th1), Th2, Th17, or regulatory T-cell.

If the co-stimulation is prevented, the T-cell will not be activated but T-cell tolerance will occur to modulate T-cell activation. One way to prevent co-stimulation is the expression of the CTLA-4 protein. The CTLA-4 protein is constitutively expressed on regulatory T-cells and upregulated on other initially activated T-cells[7]. CTLA-4 is a competitor of CD28 and also has B7.1 and B7.2 as ligands[1]. When CTLA-4 is expressed on the cell membrane it prevents co-stimulation and thereby activation of the T-cell. Moreover, the CTLA-4 protein reduces the expression of the B7.1 and B7.2 by the APC molecules via trans endocytosis, thereby preventing the activation of other T-cells. Next to the prevention of activation of T-cells, CTLA-4 is also able to inhibit IL-2 production. A decreased IL-2 production prevents

cell cycle progression by reducing cyclin production. This results in inhibition of T-cell proliferation. Moreover, CTLA-4 inhibits T-cell differentiation [7].

Another important protein for the regulation of T-cell activation is the PD-1 protein. This protein is present on apoptotic T-cells and in low levels on double-negative T-cells in the thymus, activated natural killer T-cells, B cells, monocytes, and immature Langerhans' cells and this protein is able to reduce the activation of T-cells[8]. The PD-1 protein binds to its ligands PD-L1 and PD-L2, which are both members of the B7 protein family[9]. PD-L1 is, especially during inflammation, expressed by activated T-cells, B-cells, macrophages, dendritic cells, and some epithelial cells, while PD-L2 is expressed by macrophages, dendritic cells, and mast cells. Like the binding of CTLA-4 to its ligand, PD-1 binding to the PD-L1 or PD-L2 ligand leads to inhibition of T-cell proliferation, survival, and cytokine production [10]. PD-1 protein as well as CTLA-4 acts via inhibition of the phosphoinositide 3-kinase-AKT (PI3K-AKT) pathway. Although they interact with the same pathway, it is thought that the CTLA-4 protein suppresses T-cell activation earlier on in the immune response [11].

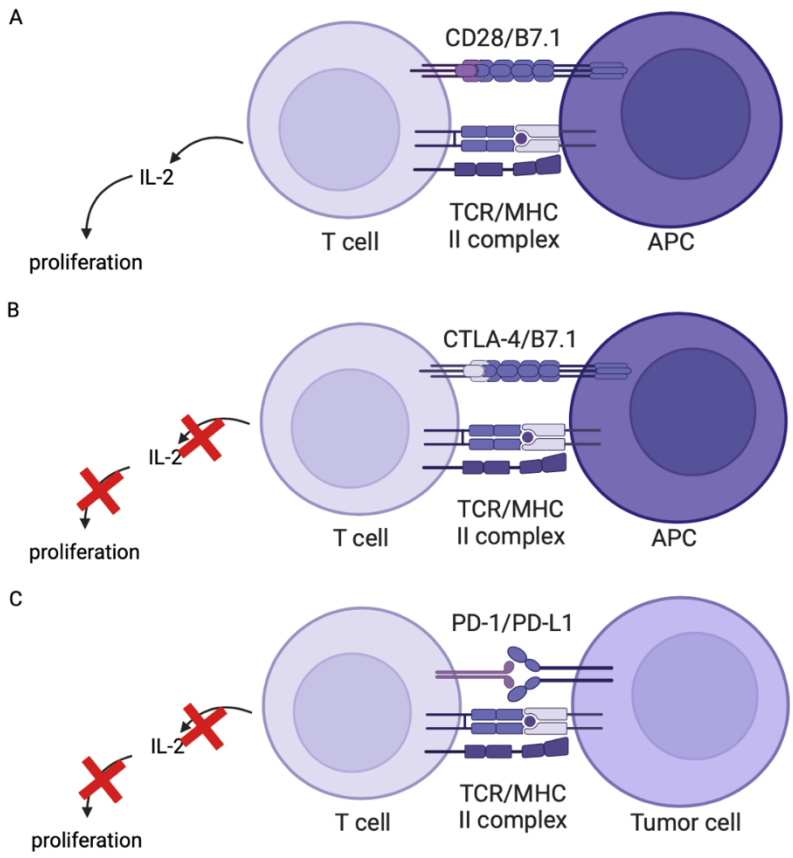

Figure 1 T-cell activation and its inhibitory signaling. *A. TCR-MHC complex needs a co-stimulatory signal of for example CD28/B7-1 to activate the T-cell. Active T-cells release IL-2, resulting in the proliferation of the T-cells. B. Binding of the CTLA-4 protein to the B7.1 ligand results in inhibition of T-cell activation. This inhibition decreases the release of IL-2 and reduces proliferation. C. The binding of PD-1 to PD-L1 has the same effect. Figure created with Biorender.*

Changes in checkpoint proteins in tumor microenvironment

Evasion of the immune system is one of the key points for cancer cells to facilitate rapid proliferation and expansion[2]. In the tumor microenvironment, there is an increased number of immunogenic molecules present over a long time. Therefore, a prolonged activation of T-cells via TCR stimulation is present, due to the chronic inflammatory condition, which leads to exhaustion of the T-cells[12]. This results in the upregulation of inhibitory molecules such as PD-1 and CTLA-4, to prevent their proliferation[13]. Moreover, the tumor cells express transforming growth factor (TGF)-β to promote the differentiation of T-cells to regulatory T-cells which express CTLA-4. This leads to an increase in CTLA-4 expression and thus more tolerant inactivated T-cells. Furthermore, the exhausted T-cells secrete reduced concentrations of cytokines, which are important for the regulation of the immune response [12].

Besides the upregulation of PD-1 and CTLA-4 by the T-cells, the hypoxic surrounding in the tumor microenvironment leads to more expression of the PD-L1 tumor cells. The increase of PD-L1 upregulation is reached via activation of the PD-L1 promotor. The exact pathways and genomic mutations resulting in the increased promotor activation differ between the various tumor types[8]. One way to induce the PD-L1 expression on the cell surface is the result of pro-inflammatory cytokines like IFN-γ and IL-6 expression of activated T-cells. Another way to increase the PD-L1 expression is the upregulation of Janus kinase 2 (JAK2)[14]. The upregulation of PD-1 by T-cells and PD-L1 by the tumor cells results in more PD-1/PD-L1 interaction. Therefore, the T-cells do not respond to TCR stimulation, which impairs the ability of the T-cell to kill cancer cells. The reduced number of T-cells results in a decreased production of inflammatory cytokines and cell survival proteins by the T-cells in the tumor microenvironment[13]. Thereby the tumor is able to escape an attack of the immune system and able to continue growing[12]. So, PD-L1 expression is negatively correlated with prognosis.

Aside from the inhibition of T-cell activation, the PD-L1 protein is also responsible for the resistance of the tumor cell against T-cell cytotoxicity. The PD-L1 expression protects the tumor cell against the pro-apoptotic signals of the T-cell by enhancing core survival pathways[8,15]. Moreover, PD-L1 contributes to tumor progression by modulating the glucose metabolism. The modulation results in a higher glucose uptake out of the tumor microenvironment, which leads to a decreased glucose concentration in the surrounding of the tumor cell. This results in lower glucose uptake by the activated T-cells, which rely on aerobic glycolysis for energy production, thereby inhibiting the cytotoxic effects. All in all, the tumor microenvironment is adapted to escape an attack of the immune system.

Effect of ICI treatment

ICI treatment has become one of the most important therapies to treat various types of cancers[16]. ICI treatment is an intravenously administered therapy. Ipilimumab, a CTLA-4 antibody, was the first US Food and Drug Administration (FDA) approved ICI treatment and was approved for metastatic melanoma. As of April 2022, 9 ICI agents are approved by the FDA as a separate treatment, as well as in adjuvant and neoadjuvant settings. ICIs are approved for a large range of tumors such as melanomas, tumors of the upper gastrointestinal tract, and lung cancer. The treatment is more effective in tumors with high tumor immunogenicity, defined as the ability of the tumor to induce a host immune response that can prevent its growth[17]. Examples of tumors with low tumor immunogenicity and thus low response to ICIs are brain and pancreatic tumors. Tumor immunogenicity and thereby ICI response is affected by factors such as tumor antigen expression, tumor-infiltrating cytotoxic T-cells, and the tumor microenvironment. Nowadays, half of the patients with solid cancer are eligible for treatment with one or multiple ICIs[17].

ICIs use the patient's own immunity to recognize and eliminate the tumor cells. The ICIs used as standard care are anti-CTLA-4, anti-PD-1, and anti-PD-L1[2,16]. As described earlier, immune checkpoint proteins modulate the T-cell immune response at different levels, which makes it possible to combine anti-CTLA-4 and anti-PD-1 therapies. CTLA-4 inhibition is thought to act as an “early responder” because it affects the T-cell initiation, while PD-1 and PD-L1 are thought to act as a “late responder” because their action is on the expansion and maintenance of T-cells [16,18]. All three agents result in increased activation of cytotoxic T-cells among other cells. These T-cells are major players in killing the tumor cells, via granule exocytosis and death ligand/death receptor system [19]. During granule exocytosis, the cytotoxic T-cells release the content of their granules, which consists of perforin and granzymes[20]. The death ligand is released by the cytotoxic T-cell and is able to bind to the death receptor present on the cell membrane of the tumor cell. Both pathways, granule exocytosis and death ligand/death receptor, trigger programmed intracellular events in the targeted tumor cells resulting in apoptotic cell death of the tumor cell. Apoptosis of the tumor cells results in a significant increase in disease-free survival and overall survival, both in adjuvant settings and in palliative care [20].

Inhibition of PD-L1 has not only an effect on the PD-1 protein of T-cells but also its individual effect on dendritic cells. In the dendritic cells, it leads to more unbound B7.1 molecules to improve the priming of T-cells, which is needed for the introduction of the adaptive immune response [18]. Moreover,

expression of the PD-1 protein in macrophages leads to increased tumor growth by inhibiting phagocytosis. Thus, it is also possible that PD-1 inhibitors also have a positive effect on the functions of the macrophages, however it is not confirmed yet[21]. So, inhibiting the PD-L1 has most likely multiple effects. Overall anti-CTLA-4, anti-PD-1, and anti-PD-L1 can induce the immune system to beat the cancer cells.

Side effects of ICIs

Despite the clinical benefits of ICI use for patients, the overactivation of the immune system can lead to several side effects. ICIs modulate the non-specific activation of T-cells; therefore, it is not surprising that they can lead to irAE[4]. Despite we know that the occurrence of irAE is related to overactivation of the immune system, the exact pathophysiology is not fully understood. CTLA-4 treatment shows different effects than PD-1/PD-L1 treatment, which may result from the different mechanisms of T-cell inhibition they interact with[18]. It is shown that patients treated with the combination therapy of CTLA-4 and PD-1/DP-L1 have more irAE, which can affect every organ. The severity of the irAE can be graded from one to five; grade one side effects are mild and are only clinical or diagnostic observed, grade two are moderate and limit activities of daily living, grade three are severe and disable or limit self-care activities of daily living but are not life-threatening, grade four are life-threatening, and grade five are fatal[17]. Most adverse events develop within the first twelve weeks of therapy as a result of short-term exposure[22], but there are case reports available of patients who developed irAE after six months of discontinuation of the drug. The irAEs due to short-term exposure are also called acute irAEs. Dermatitis, pruritus, thyroiditis or hypothyroidism, colitis, hepatitis, and arthralgia are the most seen acute side effects. The majority of these irAEs can be effectively treated with steroid drugs, however, in some patients worsening of symptoms with severe morbidity and mortality are described despite adequate immune suppressive treatment. The irAE guidelines of the European Society of Medical Oncology suggest by grades one and two to withhold ICI treatment until the side effects are dissolved. If the side effects are grade three or four, the patients need to be treated with immunosuppressive to reduce the side effects. Moreover, for grade four side effects it is advised to permanently discontinue ICI treatment[17]. Some immune-related side effects are long-term and are still present at least three months after finishing the ICI treatment[23]. Although cardiovascular-related side effects are rare, they result in one of the highest numbers of fatal events[11,22]. The documented cardiovascular side effects are myocarditis, pericarditis, arrhythmias, coronary artery disease, and left ventricular dysfunction[17].

Atherosclerosis pathogenesis

Atherosclerosis is a cardiovascular disease whose origin can be influenced by multiple factors such as diet and genetic predispositions. It is a chronic inflammation in the intima of a vessel wall that narrows the lumen of the vessel. An atherosclerotic plaque consists of a (necrotic core) of immune cells, cholesterol crystals, and plasma lipoproteins, which are surrounded by a fibrous cap[24]. The cap consists of layers of smooth muscle cells embedded in a matrix of collagen and elastin which is produced by the smooth muscle cells. The formation of an atherosclerotic plaque is initiated by the accumulation of plasma lipoproteins, such as low-density lipoprotein (LDL) and cholesterol in the subendothelial space of the vasculature at sites with the disturbed flow and dysfunctional endothelial cells (Figure 2)[24]. There, the endothelium is more permeable for the plasma lipoproteins [25]. These proteins are prone to accumulate in the vessel wall, where they become oxidized. The accumulation and oxidation of the lipoproteins leads to the ability of the lipoproteins to activate the endothelium[26]. Activated endothelium expresses adhesion molecules and pro-inflammatory cytokines. The adhesion molecules drive the attraction of circulating monocytes and T-cells[24]. The pro-inflammatory cytokines in the lesion lead to the differentiation of the monocytes to macrophages [26]. Furthermore, Plasma lipoproteins are able to activate the vascular smooth muscle cells present in the plaque. Like endothelium activation, this activation leads to chemokine signaling and adhesion molecule expression, which induce the attraction of immune cells.

Next to the activation of the endothelium, the plasma lipoproteins can also be taken up by macrophages, leading to the activation of these macrophages. The lipoproteins can be endocytosed in their native form or their oxidated form such as oxLDL[27]. Activated macrophages produce oxygen radicals, nitrogen radicals, proteases, and pro-inflammatory cytokines. The production of these molecules leads to the attraction of monocytes, T-cells, and LDL from blood circulation. Furthermore, it leads to the activation of other immune cells such as the T-cells. The upregulation of proteases by the activated macrophages leads to the destabilization of the plaque[28]. Besides the activation of the macrophages, the endocytosis of oxLDL by the macrophages or by vascular smooth muscle cells leads to the transformation of macrophages or vascular smooth muscle cells to foam cells [24]. Research has shown that foam cells have pro-atherosclerotic properties and promote the appearance of new oxLDL which leads to activation of other macrophages and attraction of immune cells[28]. Multiple factors in atherosclerotic plaques, such as oxLDL and cholesterol, are shown to promote apoptosis, which can increase inflammation by the inappropriate clearance of the apoptotic bodies. This inappropriate efferocytosis results in the formation of a necrotic core within the plaque. The visual circle of endocytosis of oxLDL, apoptosis, and activation of inflammatory cells results in the thickening of the fatty streak lesion and thereby obstruction of the vessel lumen.

As described earlier, T-cells can be activated by the macrophages. Multiple types of T-cells are detected in plaques; cytotoxic T-cells, Th1, Th2, Th17, and the regulatory T-cells. Th-1 cells produce pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α). IFN-γ enhances the recruitment of macrophages and T-cells and inhibits the formation of the vascular smooth muscle cells and TNF-α promotes leukocyte enhancement cytokine production and damaging of the endothelial cells. Th1-cells are therefore pro-atherosclerotic[24]. Regulatory T-cells are found to be protective against atherosclerosis development, due to their production of TGF-β and interleukin-10 (IL-10)[26]. TGF-β inhibits the activation of non-regulatory T-cells and macrophages and promotes the proliferation of vascular smooth muscle cells. IL-10 is able to reduce Th1 differentiation and prevents cytokine release by macrophages and T-cells[13]. The role of Th2, Th17, and cytotoxic T-cells within plaque formation is less clear. Th2 cells secrete, among other things, IL4 and IFN-γ, which both have an opposite effect within atherogenesis[24].

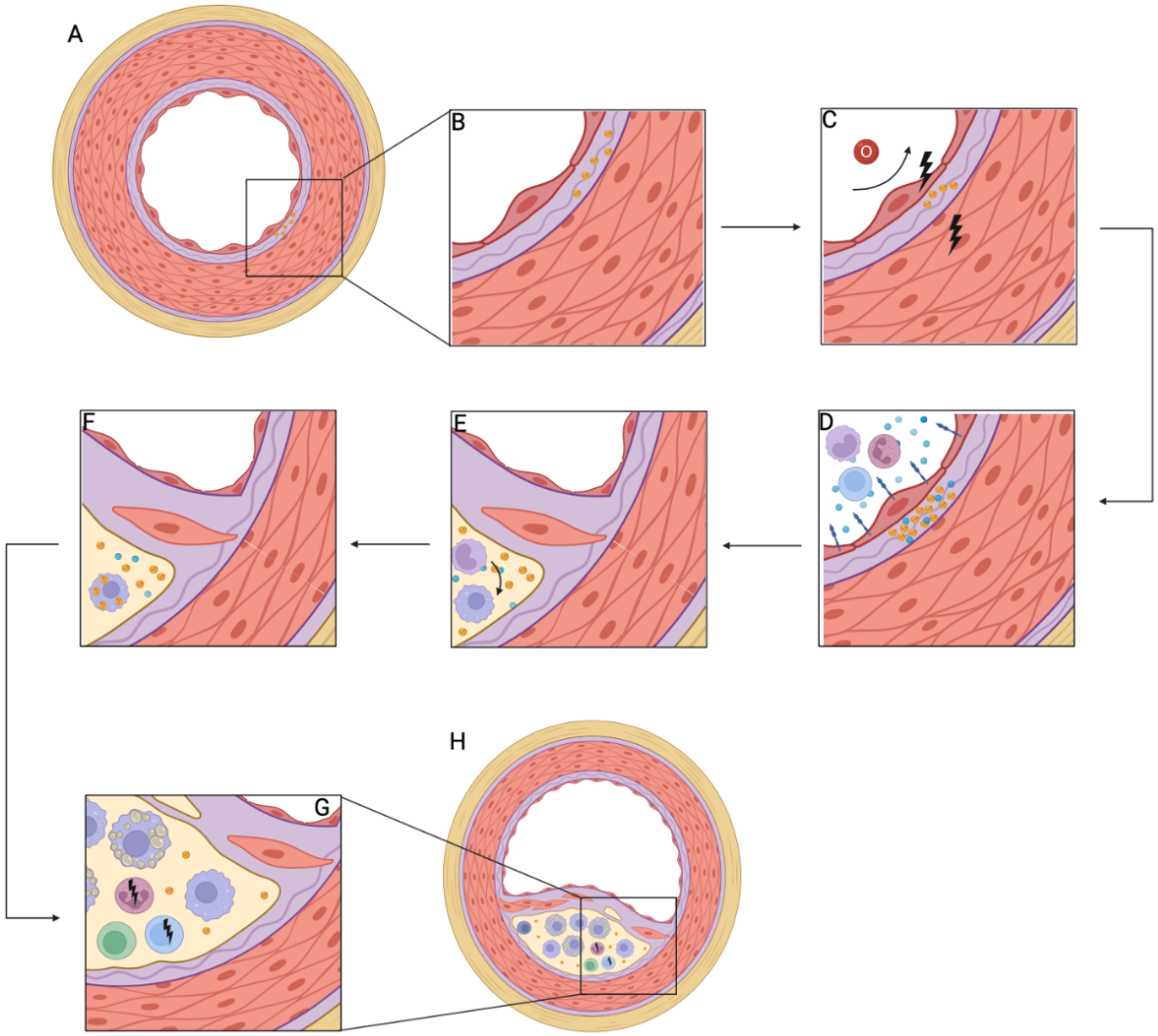

Figure 2 The pathogenesis of atherosclerosis. *A. An overview of a blood vessel wall with some LDL-particles (yellow dots) in the subendothelial layer. B. A close-up of a part of the same vessel wall. C. The oxidation of those LDL-particles results in the activation of the endothelial cells and smooth muscle cells. D. The expression of adhesion molecules and release of pro-inflammatory cytokines lead to the adhesion of different immune cells, which enter the subendothelial layer. E. These cytokines promote the differentiation of monocytes to macrophages. F. These macrophages take up the LDL particles which result in the formation of foam cells in the subendothelial layer. G. A progressed plaque, which contains a diverse type of migrated by foam cells activated immune cells and a lot of lipid particles. H. An overview of the plaque in the subendothelial layer of the vessel wall. The figure is created in Biorender.*

ICI treatment effects on atherosclerosis

As described earlier, an association between ICI use and the progression of atherosclerosis is found. To study the effect of immune cells on the development of atherosclerotic plaques due to ICI use, there are multiple animal studies performed with the inhibition of checkpoint proteins. All those animal studies used mice models that were prone to develop atherosclerotic lesions, such as the Apolipoprotein E knock-out ($Apoe^{-/-}$) or LDL receptor knock-out ($Ldlr^{-/-}$) mice. These mice were either treated with anti-CTLA-4 or anti-PD-1/PD-L1 or the CTLA-4 or PD-1/PD-L1 genes were knocked out.

One of the mice studies that observed the effect of ICI use on atherosclerosis, was performed by Poels *et al.* [5]. First, the 2-deoxy-2-[fluorine-18]fluoro-D-glucose (^{18}F-FDG) uptake in the aorta of 15-week-old $Apoe^{-/-}$ mice treated twice a week with anti-CTLA-4 and anti-PD-1 of placebo for four weeks was determined. No difference was observed in ^{18}F-FDG uptake between the ICI and placebo groups. Mice were sacrificed after 17 weeks, and no difference in aortic atherosclerotic plaque size was observed between the ICI and placebo groups. Secondly, the researchers performed flow cytometry of the aorta lesions of those two groups, which showed no difference in the inflammatory

cell content. These results together suggest that short-term ICI treatment does have major effects on vascular or systemic inflammation in hyperlipidemic mice. For further investigation of the effect of ICI on the immune response, 12-week-old Ldlr$^{-/-}$ mice were fed with 0.15% cholesterol in combination with twice-a-week anti-CTLA-4 and anti-PD-1 for five weeks. There was no difference observed in the number of B-cells, myeloid and dendritic cells. However, comparing the T-cell population of the treatment group and the control groups showed major changes. ICI treatment resulted in an increase in Th-cell and cytotoxic T-cell numbers and a decrease in naïve T-cell numbers. These changes upon short-term treatment are reflective of an activated T-cell profile. Looking at the atherosclerotic plaques in ICI and control-treated Ldlr$^{-/-}$ mice showed us more advanced atherosclerosis in ICI-treated mice. In those plaques, the number of cytotoxic T-cells is increased, and the number of macrophages is decreased. There was no difference in smooth muscle cell and collagen content observed. Next to a difference in immune cell content and the more advanced plaque, an increased endothelium activation was also observed, by increased expression of intercellular adhesion molecule (ICAM)-1. All in all, these results showed an increased atherosclerotic phenotype in mice after short-term ICI use[5].

There are also mice studies performed that assessed the effect of either PD-1/PD-L1 or CTLA-4 inhibition or knock-out. The studies in which PD-1 or PDL-1 was knocked out showed that it resulted in higher numbers of non-regulatory T-cells and macrophages [11,13]. The higher numbers of these cells lead to more naïve T-cell migration to the (early) atherosclerotic plaque where they differentiate in pro-atherosclerotic subtypes[13]. Moreover, increased numbers of T-cells and macrophages result in higher concentrations of the TNF-α, more T-cell activation, and more activity of cytotoxic T-cells[11]. This all leads to increased inflammation and enlargement of the atherosclerotic plaque. Inhibition of PD-1/PD-L1 increases the number of activated regulatory T-cells resulting in an increased release of IL-10 and TGF-β which are athero-protective. Nevertheless, the net effect is still increased plaque size and increased T-cell infiltration. Furthermore, activation of T-cells increases the release of IFN-γ, which plays a major role in the development of atherosclerosis as described earlier[13]. Inhibition of the PD-1 expression on endothelial cells results in the reduction of immune checkpoints, IL-10, and TGF-β cytokine production. Additionally, PD-L1 has been shown to protect against apoptosis of the endothelial cells, thus treatment with ICI will lead to less protection of endothelial cell apoptosis[11]. This demonstrates that PD-1/PD-L1 inhibition will lead to reduced protection of the endothelium for atherosclerotic effects.

Blockage of CTLA-4 results in the progression of atherosclerosis, via decreasing the collagen content, increasing the intimal thickening, and increasing the necrotic core area. This progression is mainly driven by T-cell-induced inflammation, because of the absence of changes in macrophage inflammatory content[5]. Overexpression of the CTLA-4 gene in mice leads to decreased intimal thickening, reduced number of Th-cells and regulatory T-cells, and less proliferation and cytokine release by T-cells[11]. So, a reduction of the expression of this gene will have the opposite effect. Treatment of mice with a reduced CTLA-4 expression with a synthetic CTLA-4 analog results in less plaque development and reduced IFN-γ and IL-2 production. In contrast to PD-1, CTLA-4 does not affect the number of lesion macrophages and enhancement of cytotoxic T-cells[13]. All in all, it is shown that there is a positive correlation between PD-1, PD-L1, and CTLA-4 use and atherosclerosis progression.

Cardiovascular events due to atherosclerosis

Small and mild atherosclerotic plaques will normally not result in symptoms in patients. However, when the vessel is almost completely obstructed by the plaque or the thrombus on top of the plaque, it will lead to symptoms[26]. The thrombus formation can be caused by plaque rupture or erosion. By plaque rupture, the thin fibrous cap ruptures whereby the plaque content is exposed to the blood which results in thrombus formation. By plaque erosion, the thrombus formation is on top of the irregular surface of the intact fibrous cap[29]. The obstruction, by the thrombus or plaque, creates a hypoxic area behind the stenosis which may lead to a major adverse cardiovascular event (MACE). The three classical components of MACE are non-fatal ischemic stroke, non-fatal myocardial infarction, and cardiovascular death[30].

One of the observational studies that observed a high risk of cardiovascular events after ICI use is Drobni *et al.* [32]. They included two study designs in their study. The first study design was a matched cohort study in which they showed a three times increased chance of cardiovascular events (myocardial infarction, coronary revascularization, and ischemic stroke) after ICI use compared to the control. They included 2048 patients in each arm which were matched by age, history of cardiovascular events, and cancer type. The other study design was a case-crossover design, in which they included 2842 patients. The researchers used the two years before the start of the ICI treatment as the control period and the two years after the start of the ICI treatment as the at-risk period. In the two years after the start of the ICI treatment, there was a four-fold increased risk of a cardiovascular event compared with the two years before ICI treatment. Similar findings were found if the control and at-risk periods were restricted to one year before and after the start of the treatment[32]. Moreover, in clinical trials, a higher number of atherosclerotic events is observed in ICI-treated patients[6].

Previously Ma *et al.* performed a systematic review and meta-analysis to research the association between ICIs and thromboembolic events [33]. They included phase II and III randomized controlled trials that describe thromboembolic endpoints in IC versus non-ICI or ICI monotherapy versus combination therapy of PD-1/PD-L1 and CTLA-4 in cancer patients. For venous thromboembolism, they included 61 papers that describe the number of events in 29861 patients, 16708 patients in the treatment group and 13153 patients in the control group. This comparison did not result in a significant difference. Moreover, they included 49 papers that describe the number of arterial thromboembolic events in 29777 patients. The treatment group consisted of 16618 patients and the control group consisted of 13159 patients. This systematic search showed an increased risk of arterial thromboembolic events in ICI-treated patients of 155 cases in the ICI-treated group versus 72 cases in the control group. Thereby the incidence of arterial thromboembolism was 0.93% in the treatment group and 0.55% in the control group [33]. However, this review was published in 2021. With the increased prescription rate of ICI therapy and the prolonged follow-up duration, we aimed to update the previous systematic review with studies published after January 2021.

Methods

Search strategy

A systematic literature search was conducted in PubMed and Embase using relevant predefined search terms. Articles published between January 1, 2021, and August 31, 2023, are included. The full search strategy is available in the supplemental data.

Study selection

Phase II and phase III randomized controlled trials that describe thromboembolic endpoints, including embolism, ischemic stroke, myocardial infarction, and cardiovascular stroke in ICI-treated versus non-ICI-treated or mono ICI-treated versus combination therapy of PD-1/PD-L1 and CTLA-4 treated oncological patients, in their main text or supplemental data were eligible for inclusion. ICI treatments included in this systematic search are PD-1/PDL-1 and CTLA-4 inhibitors, all other ICIs were excluded as they are not part of the standard of care at this moment. Dose-escalation, case reports, case series, non-randomized studies, single-arm studies, phase I clinical trials, in-vitro studies, review articles, protocols, editorials, and letters were excluded. Moreover, articles written in other languages than Dutch or English were excluded. If institutions updated earlier trials with an accumulating number of patients or increasing length of follow-up, only the most recent study was included to prevent duplication of data.

Data extraction

Two reviewers (S.B and A.S) independently searched and examined the relevant trials for further assessment with the use of the rayyan.ai program. The following data was extracted from the included papers: NTC number, study name, first author, year of publication, trial design, trial phase, tumor

stage, tumor type, regimens administered to the treatment and control arms, number of enrolled patients, median patient age, and number of interested outcomes in both arms.

Results

Literature search and study characteristics

We identified 983 records through database searching in PubMed and Embase. After removing the duplicates and screening the titles and abstracts we retained 21 potentially interesting papers. After full-text reading and applying the inclusion and exclusion criteria, we retained three studies (Figure 3). Most of the other studies do not include any information about atherosclerotic events after ICI treatment. The characteristics of the included studies are described in Table 1. All three studies were phase III clinical trials. Two trials compared ICI treatment with non-ICI treatment and one trial compared one ICI versus 2 ICIs. All three trials included patients with a different tumor type, esophageal squamous cell carcinoma (ESCC), non-small cell lung cancer (NSCLC), and metastatic urothelial carcinoma (mUC). However, all three trials included only patients with advanced tumors. Moreover, all three trials used a different ICI, and all had a different control group. Two studies were open-label randomized trials, and one study was a double-blind randomized trial.

Incidence of atherosclerotic events

None of the studies described the number of myocardial infarctions or cardiovascular deaths in the study population. All three studies described one or more strokes in either the ICI-treated arm or in both arms. Moreover, Gettinger *et al.*[34] and van der Heijden *et al.*[35] described two thromboembolic events in each arm and one embolism in the ICI-treated arm respectively. Both, the thromboembolic events and embolism, are not further specified in venous or arterial embolism. So, no difference in the number of events was observed.

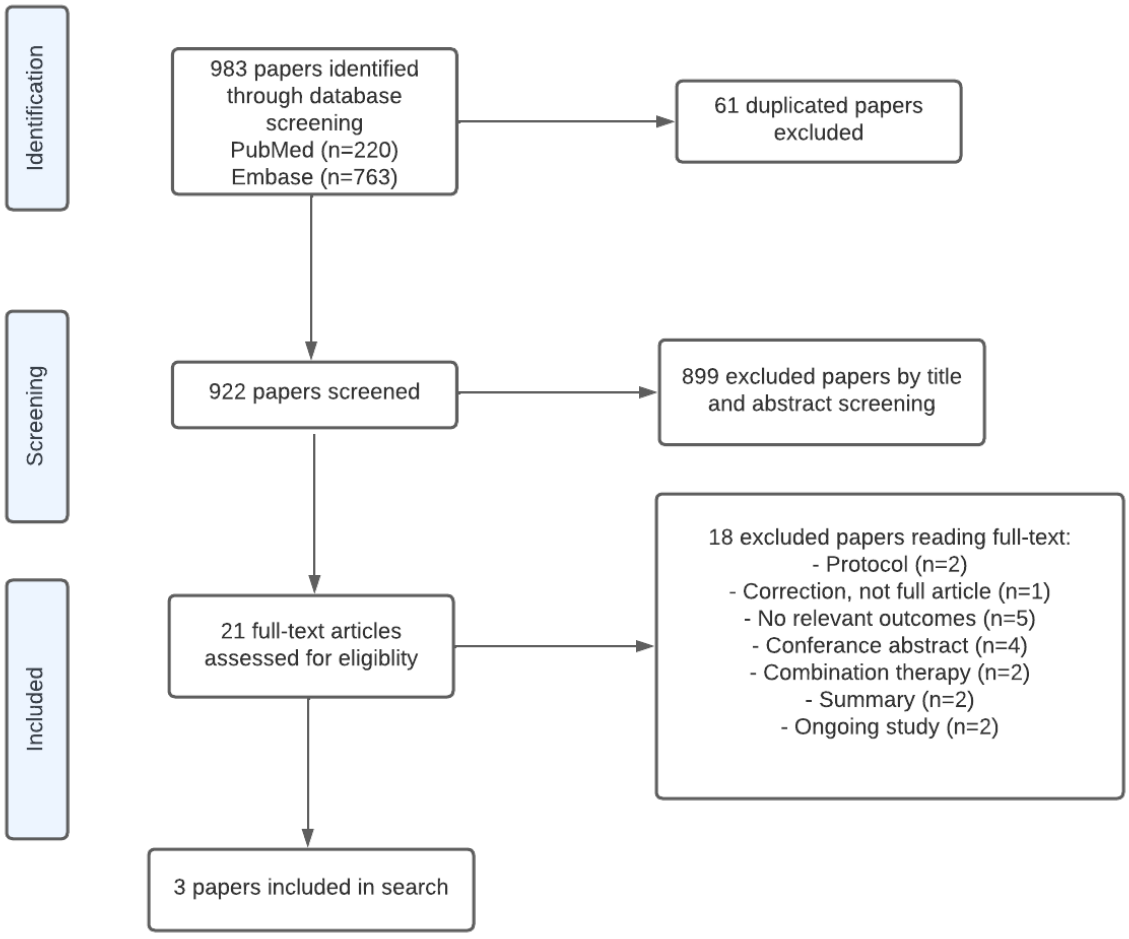

Figure 3 Flow diagram of study selection

Table 1 Study characteristics. *ESCC: esophageal squamous cell carcinoma, NSCLC: non-small cell lung cancer, and mUC: metastatic urothelial carcinoma*

Study	First author	Publication year	design	phase	tumor stage	tumor type	treatment of control and treatment arm	Patients enrolled	median age (years)	outcome
NCT02520453[36]	Park, S	2022	Double-blinded randomized	II	Advanced	ESCC	Placebo vs durvalumab	86	65	one stroke in each arm
NCT02785952[34] Lung-MAP (S1400)	Gettinger, S	2021	Open label randomized	III	Advanced	NSCLC	Nivolumab vs. nivolumab combined with ipilimumab	275	67.5	two strokes in Nivolumab + ipilimumab arm and two thromboembolic events in each arm
NCT02302807[35] IMvigor211bo	van der Heijden, S	2021	Open label randomized	III	Advanced	mUC	Chemotherapy vs. atezolizumab	931	67	two strokes and one embolism in atezolizumab arm

Discussion

Therein this review showed that there is preclinical and clinical evidence that ICI treatment, despite their clinical benefits, results in increased risk for atherosclerotic events. ICI treatment results in a reduction of active immune checkpoints and thereby increases T-cell activation and recruitment of immune cells. These cells have a more pro-inflammatory profile and produce pro-inflammatory cytokines such as IL1, IL6, and IFN-γ. This ultimately results in the prevention of tumor escape from the immune system. However, the increased T-cell activation is not only present in the tumor microenvironment but also has a systemic response. During plaque formation, an increased T-cell activation leads to an increased release of cytokines and thereby an increased inflammation and plaque size. This suggests that the association between ICI use and atherosclerosis has something to do with the immune response. However, the exact underlying mechanism is still unknown.

Until now, most cohort studies that specifically observe the increased risk of atherosclerotic effects in ICI-treated patients are retrospective studies that looked at the number of events over a short period. Generally, prospective studies have a higher value, because of the reduced influence of confounding. Moreover, the performed cohort studies did not observe the long-term effects of ICIs on thromboembolic events and included a low number of subjects. In addition, atherosclerosis is not officially documented as an immune-related side effect of ICI treatment, yet. So, the number of observed atherosclerotic events during the study and follow-up time of the study are not always described in the articles, as can be seen in the systematic part of this review article. In the past years, ICIs were mainly administrated to patients in the palliative phase with an increase in overall survival of months. As we know cardiovascular disease and atherosclerosis are slowly progressive, with an increased event risk over the years, these patients may probably die from their cancer instead of developing a cardiovascular event. Moreover, it is possible that in curative settings, the follow-up period in most studies of two to three years is too short to observe thromboembolic events. This is also observed in the preclinical study performed by Poels *et al.*[5].

In addition to the review of literature and the previously described systematic review, we performed an updated systematic review of papers published between 2021 and 2023. However, only three studies described cardiovascular endpoints. There can be multiple reasons, as described earlier, why there are only a few papers of randomized clinical trials that describe the number of atherosclerotic events. If articles do not describe the number of atherosclerotic events. It is possible that no events occurred due to the short survival time of patients or the short follow-up duration of the study. We expect the number of events to be small, however, as arterial thrombotic events are not included in known irAE, underreporting of these events is a possible explanation for the low number of events in original trials.

Although incidence rates of events were low in previously described observational studies, ICI-related cardiovascular complications might become a more emerging problem in the upcoming years. This is mainly due to the high prescription rate, the significant increase in overall survival, use of ICI as an adjuvant treatment to prevent recurrence. Therefore, it is important to research the long-term effects of ICI treatment on the risk of atherosclerotic events. It is important to know if the increased risk is not only short-term or also long-term, to be able to treat those patients as well as possible. To determine the effect of ICI on atherosclerosis and arterial thromboembolic events, more research is needed. This might include prospective studies with a longer follow-up period. Moreover, the sample size should be increased to be able to observe enough clinically relevant differences regarding atherosclerotic plaques.

Although we know that the association between ICI treatment and atherosclerosis has its origin in the activation of the immune system, the exact mechanism is still unknown. Therefore, it is important to unravel the underlying mechanism, to know how ICI treatment exactly leads to more atherosclerotic events. Techniques that can be used to get more knowledge about the underlying biological mechanism are (spatial) proteomics and RNA sequencing, to discover changes in the protein and gene expression. If the biological mechanism is known a treatment can possibly be developed to protect patients from the increased risk. This treatment should be able to reduce the risk for atherosclerotic events, via reduced plaque formation, without reducing the effect of ICIs to treat the

tumor. Moreover, there is a need to identify those patients who receive ICI who are at high risk of developing accelerated atherosclerosis and/or arterial events due to therapy. To identify this group, translational and prospective clinical studies are needed to elucidate the inflammatory and immune biomarkers linked to ICI-associated atherosclerosis. If the pathways involved in developing atherosclerosis and arterial thrombotic events are more studied, a possible target for treatment could be identified. This subgroup should be observed carefully and should be treated to reduce the risk if a treatment is available. Furthermore, we need to look further into the effect of the increased risk due to ICI treatment in patients who have already an increased risk due to other factors such as hypertension, diabetes mellitus, and smoking. One retrospective study already observed more atherosclerotic effects in ICI-treated patients with hypertension than in ICI-treated patients without hypertension [37]. They did not observe the same effect for other risk factors, such as diabetes and smoking. To conclude, it is suggested that ICI use leads to an increased risk for atherosclerotic events and that this association is most likely due to increased activation of the immune system, mainly driven by T-cells.

Taking the data about ICI-associated atherosclerosis into account, more research is needed to evaluate whether ICI treatment is associated with an increased risk of developing arterial thrombotic events due to the acceleration of atherosclerosis. Moreover, we should unravel the pathogenesis of ICI-related atherosclerosis, to be able to prevent arterial thrombotic events in cancer survivors.

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Supplemental data

PubMed search strategy:

(randomized controlled trial[pt] OR controlled clinical trial[pt] OR randomized[tiab] OR placebo[tiab] OR clinical trials as topic[mesh:noexp] OR randomly[tiab] OR trial[ti] NOT animals[mh]) AND (immune checkpoint inhibitors[MeSH Terms] OR Immune Checkpoint Inhibitor [tiab] OR Immune Checkpoint Blockers[tiab] OR Immune Checkpoint Blockade[tiab] OR Immune Checkpoint Inhibition[tiab]) OR PD-L1 Inhibitor[tiab] OR Programmed Death-Ligand 1 Inhibitors[tiab] OR PD-1 Inhibitor[tiab] OR Programmed Cell Death Protein 1 Inhibitor[tiab] OR anti-PDL1[tiab] OR anti-PD1 OR CTLA4[all fields] OR “cytotoxic T-lymphocyte-Associated Protein 4”[all fields] OR “CTLA-4 antigen”[MeSH])

Embase search strategy:

([Controlled Clinical Trial]/lim OR [Randomized Controlled Trial]/lim) AND (('immune checkpoint inhibitor'):ti,ab,kw OR (('immune checkpoint inhibitor'):ti,ab,kw) OR (('immune checkpoint protein'):ti,ab,kw) OR (('cytotoxic T lymphocyte antigen 4 antibody'):ti,ab,kw) OR (('programmed death 1 ligand 1'):ti,ab,kw) OR (('programmed death 1 receptor'):ti,ab,kw)